Anti-*Candida* Activity of Killer Toxins from the Yeast *Williopsis mrakii*

VALERIE J. HODGSON BSc. (HONS).

THIS THESIS IS PRESENTED TO DUNDEE INSTITUTE OF TECHNOLOGY IN PARTIAL FUFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF MOLECULAR AND LIFE SCIENCES,
DUNDEE INSTITUTE OF TECHNOLOGY IN COLLABORATION
WITH GLAXO GROUP RESEARCH.

AUGUST 1993.

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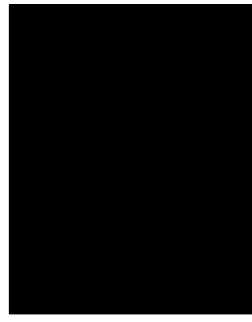

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Acknowledgements

I wish to thank my supervisor Dr. Graeme Walker for his advice throughout this project and extend my sincere gratitude to Dr. David Button for his constructive criticism of this manuscript and support both as an undergraduate and postgraduate. The cooperation and advice of the technical staff of the Department of Molecular and Life Sciences is also greatly appreciated. I would also like to express my thanks to Dr. David Ross for the access given to his personal computer.

I would like to express my sincere gratitude to Glaxo Group Research for the financial support given to this project, and myself. The understanding and advice of Dr. Russel Williamson is also gratefully acknowledged.

Special thanks must be given to close friends for their continued support, especially to Dr. Steve Millam for his time, encouragement and liquid refreshments without which the completion of this thesis would not have been possible. I would also like to extend my thanks to Anne M^{c}Leod for the time spent proof-reading and checking references and her artistic talents are greatly appreciated.

Finally, I will always be indebted to my family for their continuing support, encouragement and patience in all I do....I made it!

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Abstract

Killer yeasts secrete toxins which are lethal or inhibitory to sensitive yeasts but to which they themselves are immune. *Williopsis mrakii* is a killer yeast but very little is known about the physiology of the organism and the characteristics of its toxin. This research was concerned with an investigation into killer toxin production by *W.mrakii*, purification of the killer factor and its action against medically important *Candida* species.

A total of 30 presumptive killer yeast strains were tested *in vitro* for killing activity against a range of sensitive yeast strains, including clinical isolates of *Candida*, using a standard agar diffusion bioassay. Several yeasts, of the genus *Pichia*, showed widespread activity against the sensitive strains assayed but *W.mrakii* was demonstrated as showing the greatest anti-*Candida* activity.

Crude *W.mrakii* killer toxin showed a differential killing action against strains of *Candida* isolated from clinical specimens. No direct correlation between the site of isolation and susceptibility to the killer toxin was found. The status of the host and species-type would also contribute to the toxin-receptor interaction. It was found that at critical concentrations the killer factor of *W.mrakii* exerted a greater effect on stationary phase cells than cells from an exponential phase of growth. At low concentrations, the killer toxin produced a fungistatic effect on sensitive yeasts but at higher concentrations there was evidence to suggest that membrane damage accounted for the fungicidal effects of the killer factor. The cidal nature of the toxin was reflected in a rapid decrease in sensitive cell viability.

W.mrakii is a 'microaerophilic' yeast with simple nutritional requirements. In a gently agitated system the killer factor was produced in a minimal medium as a consequence of cell growth and its activity reached constant levels as the cells entered stationary phase. Toxin activity was lost at several stages during processing of the culture supernatants because of membrane-binding and losses to the ultrafiltrate. PhastSystem analysis and gel filtration chromatography suggested that the active toxin molecule was an acidic polypeptide with a molecular weight of 1800-5000 Da. Rapid purification of the toxin using FPLC techniques was hampered by problems in accurately assessing toxin activity in the presence of high salt concentrations. This was overcome by the development of a novel colourimetric assay of toxin activity.

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CHAPTER ONE

Introduction

1.1 The Killer Phenomenon

Antagonism in microorganisms was probably first reported in 1877, by Pasteur and Joubert, who observed the inhibitory effect of bacteria isolated from urine on *Bacillus anthracis*. Subsequently a wide range of natural antimicrobial substances have been successfully characterised which include antibiotics, bacteriolytic enzymes and bacteriocins. A similar antagonism was first observed in laboratory yeast strains by Bevan and Makower (1963). They defined three phenotypes of the yeast *Saccharomyces cerevisiae*: killer, sensitive and neutral. When killer and sensitive cells were grown together in the same broth or agar, a large proportion of the sensitive yeast were killed. No such killing occurred with incubations of neutral cells with either killer or sensitive strains. Killer yeasts kill sensitive cells by secreting into the medium a toxic proteinaceous factor (Woods and Bevan, 1968; Bussey, 1972) to which the killers themselves are immune.

To date, killer yeasts have been reported in strains of several genera including *Saccharomyces, Candida, Cryptococcus, Debaryomyces, Hansenula, Kluyveromyces, Pichia* and *Torulopsis* (Philliskirk and Young, 1975; Stumm *et al.*, 1977), *Ustilago* (Kandel and Koltin, 1978), *Rhodotorula* and *Trichosporon* (Morace *et al.*, 1984), *Hanseniaspora* (Radler *et al.*, 1985), *Williopsis* and *Zygowilliopsis* (Kazantseva and Zimina,1989; Vustin *et al.*, 1991) and *Zygosaccharomyces* (Radler *et al.*, 1993). Further reports have addressed the question of the frequency of occurrence and the range of specificity of the toxins produced. Studies of standard laboratory stocks of *S.cerevisiae* and of collections of commercial strains have demonstrated a high frequency of killer yeasts (Naumova and Naumov, 1973; Philliskirk and Young, 1975) presumably because of common parentage and extensive in-breeding of such strains. However, the frequency in the natural habitat is higher still and strains have been isolated from fruit, mushrooms, decaying plants and soils (Stumm *et al.*, 1977) and beer and wine fermentations (Young, 1982; Shimizu *et al.*, 1985). Starmer *et al.* (1987) showed there were marked differences in the incidence of killing activity seen in yeast species from communities associated with the decaying fruits and stems of cacti. Further cross reaction studies showed that a limited number of killer-sensitive relationships occurred within the same habitat at a particular time and locality but more frequently between yeasts from different habitats. Killer toxin production may, therefore, affect the natural

distribution of both the killer and sensitive strains and is potentially a mechanism for interference competition *i.e.* preventing a competitor from gaining access to resources (Crombie, 1947; Gill, 1974). The generally low pH activity profile of the toxins complements the natural condition of most yeast substrates during colonisation, especially that of fruit (Starmer *et al.*, 1987).

Possession of killer character could, in all likelihood, be linked to the pathogenicity of some yeast strains when it is considered that killer toxins can produce lethality in certain sensitive strains. The first detailed report of killer toxin production in pathogenic strains was of *Torulopsis glabrata* (Bussey and Skipper, 1975) but this phenomenon has since been reported in most *Candida* strains (Rogers and Bevan, 1978) and in *Cryptococcus* (Middelbeek *et al.*, 1980a). A study by Kandel and Stern (1979) demonstrated that of 236 potentially pathogenic strains of *Candida*, *Cryptococcus*, *Torulopsis* and *Trichosporon*, obtained from various hospitals and educational establishments, only 3% were found to show killer characteristics. It would appear there is no direct evidence that pathogenicity is associated with the possession of killer character and some strains, especially *C.albicans*, are more notable for their high frequency of susceptibilty to killer strains of other yeast species than for their ability to kill (Middelbeek *et al.*, 1980b; Polonelli *et al.*, 1983).

However, several factors may result in an underestimation of the number of killer and sensitive isolates identified, including:

(a) the screening methods employed being inappropriate in terms of the choice of media, incubation conditions and the indicator strains used (Kandel, 1988).

(b) the amount of toxin produced may be very low and may require concentration to aid detection (Middelbeek *et al.*, 1980b), and

(c) the presence of extracellular enzymes may destroy toxin in culture *e.g.* acid proteases secreted by *Candida* strains (Kwon-Chung *et al.*, 1985).

The apparent sensitivity of most natural microbial and fungal isolates and their inability to produce killer toxin may sometimes be based on a failure to determine optimal assay conditions and to select the appropriate complementary tester strains (Polonelli *et al.*, 1991a).

1.1.1 Classification of Killer Yeast

The classification of substances which are inhibitory or toxic can be achieved in several ways (Young, 1987), these include:

(a) the determination of their spectrum of activity against sensitive strains.

(b) the assay of their activity against mutants with known resistance to killer toxins.

(c) the determination of the biochemical nature of the toxin.

(d) the assay of the cross-reactivity of the toxin-producing strains.

The simplest and most effective method is to monitor the interaction between killer strains, testing their ability to be killers and also their immunity or their resistance to one another by cross-reaction studies. The rationale behind such a system is that killers are immune to their own toxin, thus, if one killer strain kills another then their toxins must be biochemically distinct. Any differences observed are consequently reflected in both the nature of the toxins produced and the immunity systems possessed by each strain (Rogers and Bevan, 1978). Young and Yagui (1978) classified 20 killer yeasts in this manner, identifying 10 distinct types of spectrum of killer activity (K1-K10) and 10 different patterns of resistance (R_a-R_j). Subsequent studies (Rogers and Bevan, 1978; Henschke, 1980) are substantially in agreement with these findings. Whilst the studies support the classification of killer yeasts by cross-reactivity they also show that different toxins have common properties, although the genetic basis of toxin production may be different (Young and Yagui, 1978).

1.1.2 Killer Systems Among Various Yeast Genera

(a) *Saccharomyces*

Since the first description of the killer phenomenon by Bevan and Makower (1963), different types of killer yeasts have been observed in *S.cerevisiae*, such as K1, K2 and KT28 killer phenotypes. The K1 killer system has been the archetype for subsequent research (Bussey *et al.*, 1990). The K1 killer toxin is a secreted, pore-forming glycoprotein which kills sensitive yeast cells by altering the permeability of the cell membrane, thereby causing uncontrolled efflux of ions across the cell surface (Pfeiffer and Radler, 1984; Bussey, 1991).

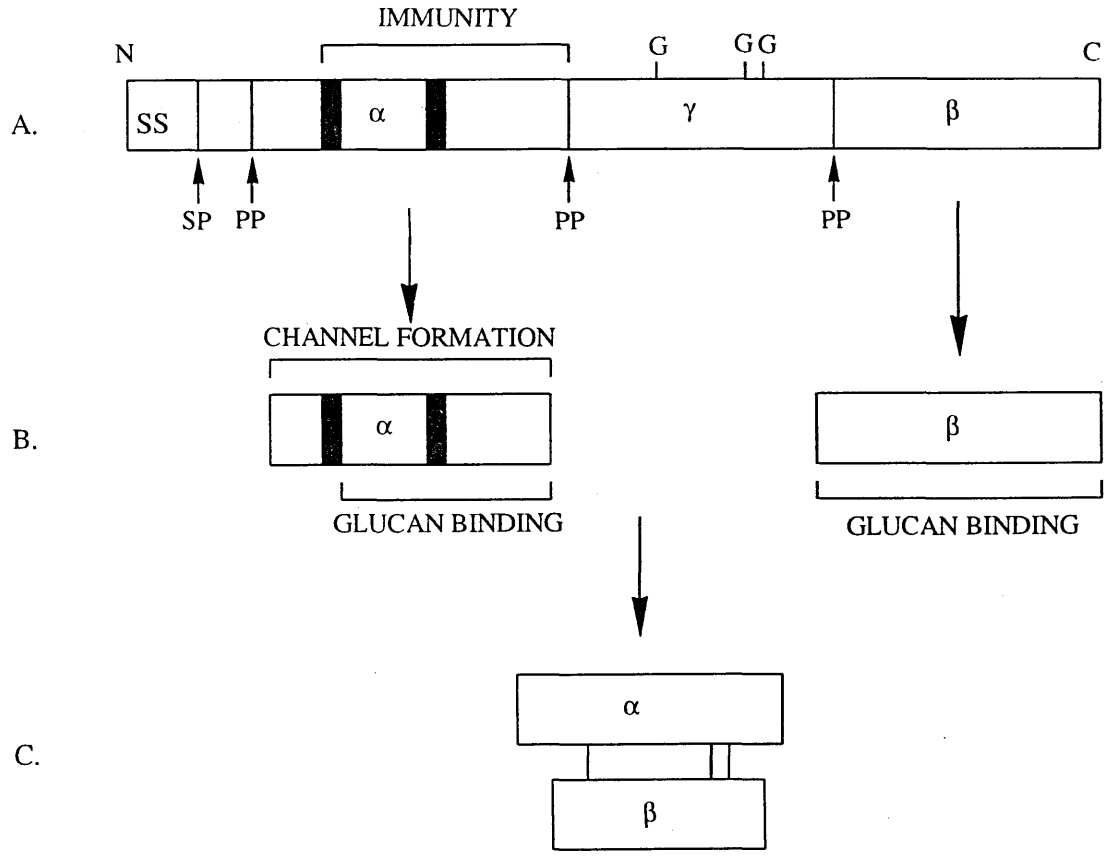
Genetic analysis revealed that the killer trait was determined by two cytoplasmically inherited, linear double-stranded ribonucleic acid (dsRNA) virus-like particles (Sweeney

et al., 1976). The larger species (3.8-5.3 kb), called LdsRNA, encodes the capsid protein and is present in most strains of *S.cerevisiae*. However, a smaller species (1.7-2.6 kb), called MdsRNA, is present in all killer strains of this type, thus, evidence strongly suggests that it determines the killer phenotype (Somers and Bevan, 1969; Bevan *et al.*, 1973; Mitchell *et al.*, 1976) and in fact encodes the toxin protein (Bostian *et al.*, 1980). This is further supported by evidence that killer cells 'cured' of their ability to kill, by incubation at elevated temperature, treatment with cycloheximide or acridine orange, lose the MdsRNA particle (Vodkin *et al.*, 1974; Young and Yagui, 1978; Cansado *et al.*, 1989).

The mature K1 killer toxin is a secreted 20-kilodalton (20 kDa) heterodimeric protein consisting of α- and β-subunits linked by disulphide bonds (Bostian *et al.*, 1984). It is derived from a precursor molecule which is extensively post-translationally processed in the yeast secretory pathway (Bussey, 1991). The toxin precursor enters the endoplasmic reticulum where the signal peptide is removed (see Figure 1.1) and the γ peptide is N-glycosylated. At a late golgi step the molecule is further processed by proteolytic cleavage (Bostian *et al.*, 1983; Bussey *et al.*, 1983). The mature toxin is then secreted via the constitutive secretory pathway to the external environment (Bussey *et al.*, 1990).

The action of this killer toxin on sensitive yeast cells, although not fully understood, is thought to be a two-stage process involving a series of specific cell surface interactions (Figure 1.2). The first stage involves binding of toxin to a (1,6)-β-D-glucan, a component of the cell wall receptor (Hutchins and Bussey, 1983). Both toxin subunits appear to participate in cell wall receptor binding. The β-subunit is necessary for glucan receptor binding, whereas the α-subunit, also involved in glucan binding, is multifunctional (Bussey, 1991). Initial cell wall binding mediates access of the toxin to a secondary receptor (**R**) on the plasma membrane where the toxin exerts a lethal effect (Stage 2) by creating a 'leak pathway'. The channel-forming domain of the toxin appears to be confined to the hydrophobic α-subunit (Zhu and Bussey, 1991) and, following an initial lag period (de la Pena, 1980) loss of ATP, K^+ and cellular metabolites follows (Bussey and Sherman, 1973; Skipper and Bussey, 1977). The toxin reduces the proton gradient across the membrane of the yeast cell resulting in the acidification of its contents (de la Pena, 1981) and cell death follows functional damage to the plasma membrane (Vondrejs, 1987). Kagan (1983) showed that a crude

Figure 1.1 - Schematic representation of the toxin precursor molecule and mature secreted toxin (Bussey *et al.*, 1990; Bussey, 1991)

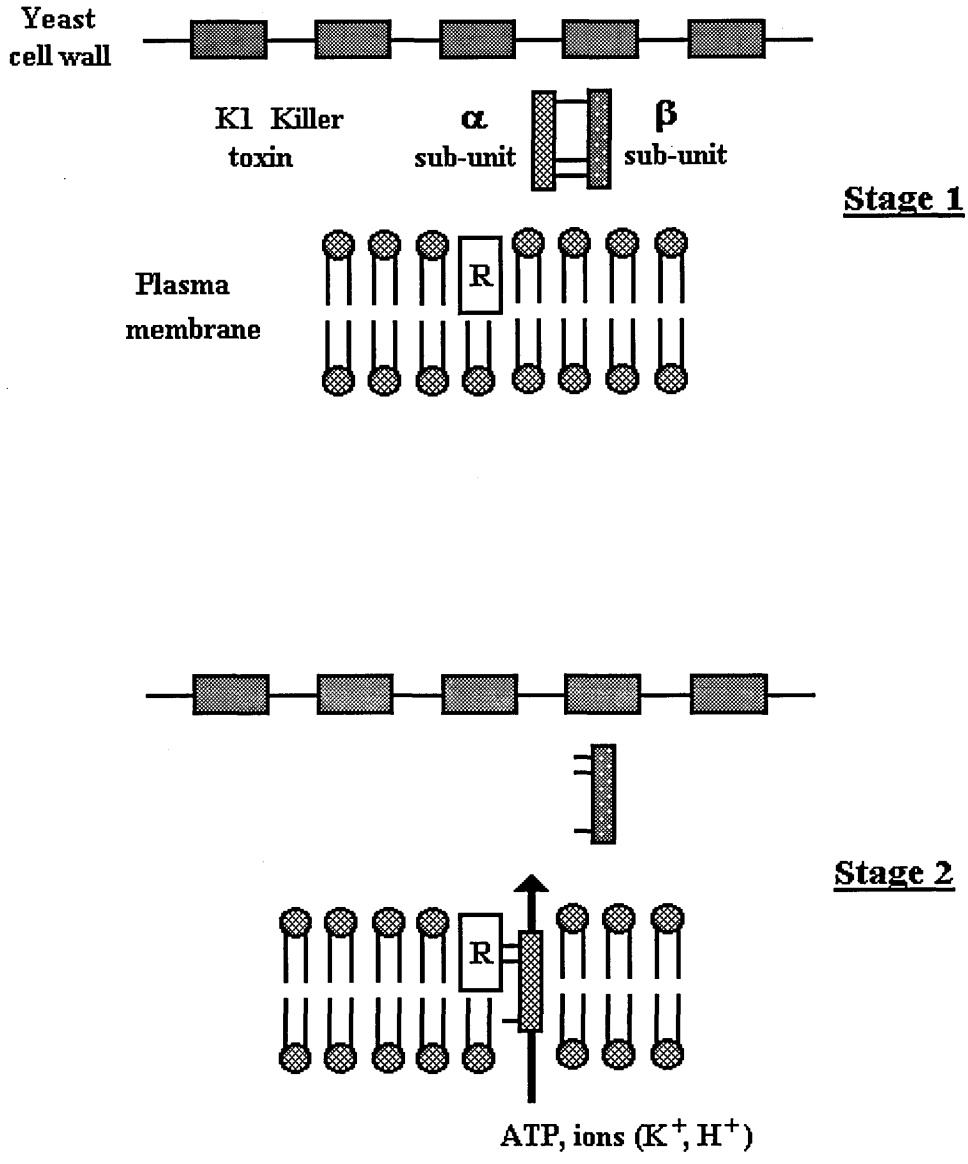


A. **Precursor molecule.** From N- to C-terminus are the signal sequence (SS) followed by α- and β-subunits separated by a glycosylated (G) γ peptide. Sites of processing by signal peptidase (SP) and proteases (PP) are shown.

B. **Toxin subunits.** α- and β-subunits which constitute the mature toxin. Two hydrophobic domains (in black) may be capable of spanning the membrane and are likely to be involved in channel formation. β-subunit is hydrophilic and involved in receptor binding.

C. **Mature secreted toxin.** α- and β-subunits are covalently linked by disulphide bonds.

Figure 1.2 - Schematic representation of the action of the K1 killer toxin of *S.cerevisiae* against sensitive yeast strains.



preparation of *P.kluyveri* killer toxin could form ion permeable channels in phospholipid bilayers, suggesting that such channels would be sufficient to explain the *in vivo* effects of *P.kluyveri* and *S.cerevisiae* killer toxins.

It is considered by Douglas *et al.* (1988) that the precursor of the mature toxin is responsible for the specific immunity of the toxin-producing strain. The precursor molecule either alters or masks the membrane receptor so that it is incapable of interacting with the α-subunit of the toxin. Alternatively the precursor could mediate the relocation or removal of the receptor so that it is no longer available for interaction.

The killer toxin, KT28, of *S.cerevisiae* 28 is a glycoprotein of molecular weight 16 kDa (Pfeiffer and Radler, 1982) and shows a degree of homology to the K2-type killer toxin (Pfeiffer and Radler, 1984). However, the toxin differs from that of other *S.cerevisiae* killer strains in that it does not bind to a glucan primary receptor but to a mannoprotein constituent of the sensitive cell wall (Pfeiffer and Radler, 1982; Schmitt and Radler, 1987). Any minor modifications to the mannan structure result in the prevention of toxin binding and confer resistance on the sensitive strain (Schmitt and Radler, 1988). KT28 killer toxin interferes with DNA synthesis or with the mitotic separation of DNA which leads to an interruption in early G2 phase of the cell cycle (Schmitt *et al.*, 1989).

b. *Kluyveromyces*

Killer toxin-like inhibitors have been reported by Lehmann *et al.* (1987) in several *Kluyveromyces* species. However, the most extensively studied has been the killer system of the yeast *K.lactis* which produces a protein toxin that inhibits growth in sensitive yeasts (Stark *et al.*, 1990). The secreted heterotrimeric protein toxin is associated with two linear double-stranded deoxyribonucleic acid (dsDNA) plasmids of 8.9 and 13.4 kb, and is active against yeast from several genera (Gunge *et al.*, 1981). The *K.lactis* toxin does not elicit leakage of K^+ and ATP (Sugisaki *et al.*, 1983) but causes an irreversible arrest of the cell cycle (White *et al.*, 1989) by an as yet undetermined mechanism. The γ-subunit appears to be the only component necessary for the arrest of proliferation since intracellular expression of this polypeptide alone mimics the action of mature toxin (Tokunaga *et al.*, 1989). Recently the α-subunit of the toxin has been demonstrated to show localised sequence similarity to a variety of plant and bacterial chitinases (Stark *et al.*, 1990; Bradshaw, 1990). It is thought to be required

for the entry of the active subunits (γ-subunit) into the sensitive cell, or may be involved in an interaction with a carbohydrate receptor on the cell wall (Butler *et al.*, 1991) in a similar manner to the K1 and KT28 toxins of *S.cerevisiae*.

Killer character determined by DNA plasmids, once unique to *K.lactis*, has recently been observed in strains of *P.acaciae* (Worsham and Bolen, 1990) and *P.inositovora* (Hayman and Bolen, 1991). The function of the linear DNA plasmids of the yeasts *S.kluyveri* (Kitada and Hishinuma, 1987) and *Saccharomycopsis crataegensis* (Shepherd *et al.*, 1987) has not been determined.

c. *Williopsis* and Others

Williopsis mrakii is an ascosporogenous, apiculate yeast found naturally in soils but which has also been isolated from swamp and creek waters. Formerly this yeast was classified in the genus *Hansenula*, as in Kreger van Rij (1984), but it now forms part of the genus *Williopsis*. For taxonomic purposes the genus *Hansenula* has been divided into two: strains with saturn-shaped spores are now termed as *Williopsis* (Barnett *et al.*, 1983; Kurtzman, 1984) and those with hat-shaped spores *Pichia*.

Killer yeasts have been reported to be widely distributed between strains of both *Pichia* and *Williopsis* (Nomoto *et al.*, 1984; Kazantseva and Zimina, 1989) and that they possess a broad range of antimicrobial activity against other yeast and filamentous fungi. One strain of *W.mrakii* (LKB 169) was isolated and partially purified by Yamamoto and co-workers (1986a). This toxin is a molecule of approximately 10.7 kDa and was thought to preferentially inhibit β-(1,3)-glucan synthesis in sensitive yeast cells (Yamamoto *et al.*, 1986b). A killer toxin of *P.anomala* (WC65) has been characterised as a glycoprotein of molecular weight 83.3 kDa which consisted of 86% protein and 14% carbohydrate (Sawant *et al.*, 1989). The toxin showed saturation kinetics at high toxin concentrations, which is indicative of a receptor-mediated mode of action (Sawant *et al.*, 1988) and it was speculated that a β-(1,6)-glucan was involved in receptor composition (Sawant and Ahearn, 1990). The toxins of *Pichia* and *Williopsis* (*Hansenula*) species are broad spectrum, displaying intrageneric activity, and they are relatively stable to a range of temperatures and pH's in comparison to the toxins of *Saccharomyces spp.* (Young, 1987).

There is no available evidence that the killer character expressed by strains of *Williopsis* and *Pichia*, or in fact *Candida*, *Debaryomyces*, *Cryptococcus*, and *Torulopsis* are dependant on plasmid encoded systems (see Table 1.1 for summary). None of the non-*Saccharomyces* yeasts studied by Young and Yagui (1978) could be cured of their killer activity by treatment with cycloheximide or ethidium bromide or incubation at an elevated temperature. Attempts to isolate dsRNA or DNA plasmids by the procedure of Gunge *et al.* (1981) proved unfruitful. This would indicate that the genetic basis for their killer character is not cytoplasmic but is chromosomally encoded.

1.1.3 Applications of Killer Yeast

(a) Advances in Fundamental Biological Research

Yeasts regulate their growth in response to numerous and different external signals. However, like higher eukaryotes, yeasts progress through a series of predetermined events which control the cell cycle and the two systems share many common features. Yeast can, therefore provide a useful model for the study of many regulatory mechanisms. The mechanisms of processing and secretion of yeast killer toxins as well as their binding and activity against sensitive cells, closely parallel the production and action processes of hormones and neuropeptides in animals (Chan *et al.*, 1979; Sossin *et al.*, 1989). The killer plasmids of *S.cerevisiae* and *K.lactis* have the potential to be used as cloning vectors for the effective secretion of expressed foreign polypeptides (Sugisaki *et al.*, 1985; Dignard *et al.*, 1991).

(b) Fermentation and Food Industries

The presence of killer yeasts as contaminants in brewing processes presents a greater threat than the presence of other wild yeast, since not only do they compete for substrate but they may eradicate the indigenous brewing strain resulting in abnormal fermentations and products of poor quality (Young and Philliskirk, 1975). Constructs, where the killer character can be conferred into strains (Vondrejs, 1987), can be used commercially for beer production (Young, 1981), wine making (Hara *et al.*, 1980; Boone *et al.*, 1990) and in the baking industry (Bortol *et al.*, 1986). Brewing strains with killer character produced beers very similar to those produced by control brewing strains, and were indistinguishable by taste (Young, 1981; Hammond and Eckersley,

Table 1.1 - The genetic basis of known killer yeasts.

GENETIC BASIS	GENUS	SPECIES	REFERENCE
ds RNA	Saccharomyces	cerevisiae	Makower and Bevan (1963)
	Hanseniaspora	uvarum	Zorg et al. (1988)
Plasmids	Ustilago	maydis	Hankin and Puhalla (1971)
	Kluyveromyces	lactis	Gunge et al. (1981)
Linear DNA	Pichia	acaciae	Worsham and Bolen (1990)
Plasmids	Pichia	inositovora	Hayman and Bolen (1991)
	Candida		Yokomori et al. (1988)
	Pichia	farinosa	Suzuki and Nikkuni (1989)
	Pichia	anomala	Kagiyama et al. (1988)
Chromosomally	Williopsis	mrakii	Nomoto et al. (1984)
Encoded ?	Torulopsis	glabrata	Young and Yagui (1978)
	Cryptococcus	laurentii	Middelbeek et al. (1980a)
	Debaryomyces	vanrijii	Young and Yagui (1978)
	Kluyveromyces	drosophilarum	Young and Yagui (1978)

1984). Crude toxin preparations have also been used direcly to protect beer from contamination by wild yeasts (Young and Talbot, 1979). Toxicity to humans of killer factor is judged as negligible and is unlikely to cause any reaction when consumed orally because of the vastly disparate pH and temperature conditions encountered (Pfeiffer *et al.*, 1988). The major disadvantage may be consumer acceptance of products involving 'killer toxins'.

The production and storage of foods is frequently compromised by growth of yeasts such as *Kloeckera apiculata* (which produces low concentrations of alcohol as an undesirable fermentation product) and *Saccharomycodes ludwigii* (which displays a high resistance to SO_2). Biological control of these undesirable yeast may be achieved using other yeast cultures which produce antifungal metabolites such as yeast killer toxins. *K.lactis*, *K.phaffii* and *K.vanudenii* proved to be effective against such contaminants (Palpacelli *et al.*, 1991) and may have potential in the food industry.

(c) Biotyping

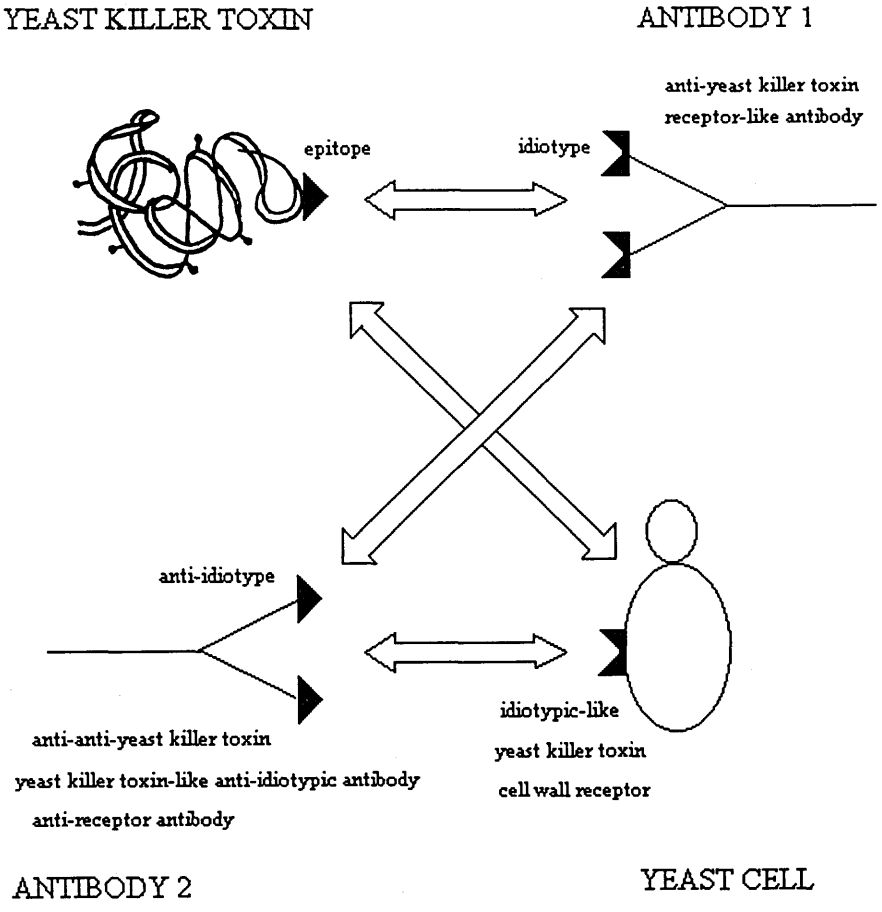
The growth inhibition produced in sensitive yeast strains by killer yeasts and their toxins has been exploited by several groups as a means of biotyping organisms to provide valuable epidemiological information concerning microbial infections in patients (Polonelli *et al.*, 1989a). A killer system was developed (Polonelli *et al.*, 1983) which grouped organisms according to their different sensitivities to a number of killer yeasts. The organisms were grouped into categories and their responses to the toxic effect of nine killer yeast were reproducible even if other characteristics were heterogenous (Morace *et al.*, 1984). Modified killer systems, adapted to the growth requirements of the sensitive strains being tested, have been used for intraspecies differentiation of *C.albicans* strains (Polonelli *et al.*, 1983) and other pathogenic *Candida* and *Cryptococcus* strains (Morace *et al.*, 1984), and to distinguish between unrelated eukaryotes (Polonelli and Morace, 1986) and bacterial isolates (Morace *et al.*, 1989). The use of the killer system provides a practical means of biotyping opportunistic yeasts and monitoring nocosomial infections. Results have been found to be comparable to serotyping studies using monoclonal antibodies and those obtained from commercial diagnostic kits (Polonelli *et al.*, 1989a).

(d) Novel Therapeutics

Polonelli *et al.* (1989b) found quantitative differences in the sensitivity of both the yeast and mycelial form of the dimorphic fungus *Sporothrix schenckii* to yeast killer toxin. Theoretically, this makes possible a therapeutic approach involving killer toxins or their derivatives in the treatment of systemic mycoses in which one morphology may be found. However, the pH and temperature lability may render yeast killer toxins unsuitable for administration orally or by injection in treatment of yeast infections (Kandel, 1988). Topical applications in the treatment of superficial lesions may be possible. Experimental infections were produced in animals by the cutaneous application of cultures of *Malassezia furfur* and *M.pachydermatis* and were treated topically with concentrated *P.anomala* killer toxin. A clear clinical improvement in the treated area occurred within 6-7 days of treatment (Polonelli *et al.*, 1986).

Another important consideration when contemplating the therapeutic use of killer toxins is the induction of pathological effects. A presumptive toxicity for cells in tissue culture was suggested (Polonelli *et al.*, 1991a) and the proteinaceous nature of the toxin is also likely to elicit an immune response from the patient (Kandel, 1988). It is hoped that natural molecules exploiting a specific physiological target, such as yeast killer toxin cell wall receptors, will create fewer adverse effects than the toxins themselves (Polonelli *et al.*, 1991a). There is evidence to suggest that certain anti-idiotypic antibodies (anti-Ids) which structurally express an internal image of the antigen (the epitope of the killer toxin) may have significant practical implications (Polonelli *et al.*, 1991b). Figure 1.3 shows the relationship between killer toxin, its cell wall receptor and resulting anti-Ids. Anti-idiotypic vaccination may be a possibility where the anti-Ids mimick the action of a yeast killer toxin *in vivo* (Polonelli *et al.*, 1991a). Yeast killer toxin anti-Ids, raised against *P.anomala,* have been shown to kill *C.albicans* cells that are sensitive to the activity of yeast killer toxin (Polonelli *et al.*, 1991b), and to protect mice when challenged with toxin-sensitive *C.albicans* (Polonelli *et al.*, 1989c).

Figure 1.3 - Receptor-mediated interactions of yeast killer toxin-like antiidiotypic antibodies (Polonelli *et al.*, 1991a).



1.2 Yeasts Pathogenic to Man

Diseases caused by fungi are collectively called mycoses, and range from very common mild infections affecting the skin, through deep cutaneous and subcutaneous infections, to acute and chronic infections of the viscera and tissues. Other than the dermatophytoses, the infections caused by yeast outnumber all other mycoses (Hurley *et al.*, 1987). The important yeast pathogens of man are *Candida* and *Cryptococcus*, and becoming increasingly important are the so-called 'major endemic mycoses', *Coccioides immitis*, *Paracoccioides braziliensis*, *Histoplasma capsulatum* and *Blastomyces dermatitidis*, which in their dominant mycelial form are infectious to humans by inhalation (Graybill, 1992).

Most of the yeasts that cause disease in man are normally commensals, exerting pathogenic effects only when the balance between the host and its indigenous flora is disrupted. The opportunity for yeasts to invade tissues is afforded by many factors, including procedures of modern medicine and therapy with antibiotics which suppress normal bacterial flora (see 1.3.2 (d)).

1.3 *Candida* and Candidosis

Amongst the yeasts, the most versatile of the pathogens are those of the genus *Candida*. Infections are caused primarily by the single yeast species *C.albicans*, but *C.kefyr*, *C.parapsilosis*, *C.tropicalis*, *C.glabrata*, *C.guillermondii*, *C.krusei* and *C.lusitaniae* also command attention as potential pathogens (Odds, 1988). Infections of virtually every tissue have been reported (Odds, 1988) but manifestations as superficial lesions are by far the most common, especially infections of the mucous surfaces of the mouth and vagina, usually termed as 'thrush'. A more serious invasive form may follow dissemination of the yeast and direct entry of yeasts into the bloodstream may occur (Shepherd, 1985a).

1.3.1 *Candida* Infections in AIDS Patients

Fungal infections are now widely recognised as common and frequently life-threatening opportunistic infections in patients with the acquired immunodeficiency syndrome (AIDS) (Sobel, 1990). Various factors have increased the numbers of immunocompromised patients, none as dramatically as the AIDS epidemic. A

progressive decrease in T-cell mediated defence mechanisms has led to an increase in mycoses such as candidosis, cryptococcosis, histoplasmosis and coccioidmycosis (Dupont, 1992). Table 1.2 shows the incidence of AIDS cases, and the number of deaths as a result, in the United Kingdom as a whole between January 1982 and January 1993. Retrospective studies have shown that 58-81% of all AIDS patients contract a fungal infection and 10-20% have died as a direct result of the contracted mycoses (Holmberg and Meyer, 1986).

The first clinical descriptions of AIDS by Gottlieb *et al.* (1981) mentioned not only pneumonia due to *Pneumocystis carinii*, but also oral candidosis and 90-95% of HIV-positive patients develop clinical lesions of *Candida* as their condition progresses (Dupont, 1992). A large array of clinical manifestations of *Candida* are seen, with the prevalence of mouth infections approaching 50% and those of the oesophagus being greater than 10% (Selik *et al.*, 1987). However, dissemination of the fungus to deep organs is considered to be rare (Odds, 1992). Klein *et al.* (1984) showed that oral candidosis is an initial manifestation in high risk patients and usually indicates a progression towards full-blown AIDS. Its use, however, as a prognostic marker for AIDS is not always straight forward and changes such as faecal dermatitis and yellowing of the toenails are seen as much earlier indications of disease progression (Mofeldt-Maanson *et al.*, 1989). The presenting symptom of a *Candida* infection should always be regarded as a possible sign of an underlying pathological process, even if it is proved later not to be HIV infection (Odds, 1992).

1.3.2 Factors Predisposing the Host to Candidosis

Candida species are strictly opportunistic pathogens which cause infection when the equilibrium between the host and yeast microflora, which normally ensures their avirulent commensal status, is disrupted. *Any* alteration in *any* microbial defence can compromise the ability of a host to withstand microbial attack (Mims, 1987). There are a large range of situations in which yeast invasion is enhanced, and these include :

(a) Natural Factors

Any person receiving medical attention is more likely to harbour yeast than 'normal' individuals and apart from the debilitating effects of many illnesses there are certain diseases and disorders particulaly associated with candidosis (Odds, 1988). Notably,

Table 1.2 - Table showing figures for known AIDS cases (and deaths) by exposure category between January, 1982 and January, 1993 (data was obtained from AIDS News Supplement, prepared and presented by the Communicable Diseases (Scotland) Unit, Ruchill Hospital, Glasgow).

Probable Acquisition of the Virus	January 1982 - January 1993			
	Male	(Deaths)	Female	(Deaths)
Sexual Intercourse				
Between Men	5333	(3356)	-	-
Between Men and Women	-	-	-	-
High Risk Partner[1]	24	(10)	49	(29)
Other Partner Abroad[2]	339	(177)	192	(71)
Other Partner UK	36	(16)	29	(17)
Under Investigation	6	(4)	-	-
Injecting Drug Use (IDU)	226	(134)	95	(54)
IDU and Sexual Intercourse	-	-	-	-
Between Men	115	(75)	-	-
Blood				
Blood Factor (Haemophiliacs)	337	(257)	5	(3)
Blood / Tissue Transfer				
- UK	19	(14)	19	(14)
- Abroad	15	(9)	30	(18)
Mother to Child	38	(17)	43	(21)
Other / Underestimated	83	(51)	12	(5)
TOTAL	**6571**	**(4120)**	**474**	**(236)**

1 - Includes men and women who had sex with injecting drug users, or with those infected by contaminated blood and women who had sex with bisexual men.

2 - Includes persons without other identified risks from, or who have lived in, countries where the major route of HIV-1 transmission is through sexual intercourse between men and women.

genital *Candida* infections have been described as common symptoms of the onset of diabetes in both men and women (Nagesha and Ananthakrishna, 1970) and diabetes has also been implicated in 5-15% of reported cases of systemic candidosis (Odds, 1988). It is proposed that the high glucose levels in the blood and tissues and the low levels of lactate in the skin (Kandhari *et al.*, 1969) may favour the growth of *Candida* in diabetics. *Candida* species are also frequently associated with endocrine disorders (Alteras *et al.*, 1969), with patients suffering from congenital disorders such as cystic fibrosis (Jenner *et al.*, 1979), cancer and haematological malignancies (Bodey and Fanstein, 1985) and increasingly with patients suffering from AIDS (see section 1.3.1). Digressions from normal physiological status may also increase the susceptibilty to fungal infections. The relative vulnerability of the young to *Candida* infections, especially of the mouth, probably stems from immature antimicrobial defences and is further increased with low birth weight , birth defects and any subsequent antibiotic treatment (Lay and Russel, 1977). Old age itself cannot be cited directly as a factor predisposing to candidosis but rather the diseases and treatments associated with old age. Vaginal carriage of yeasts is generally greater in pregnant women and they are more susceptible to vaginal candidosis. Corticosteroid effects, pH changes associated with the vagina, the concentration of vaginal glycogen and the increased receptivity of the vaginal epithelium are all considered as single or concerted factors which increase their vulnerability to infection (Sobel, 1986).

(b) Dietary Factors

An excess or deficiency of specific foodstuffs in an individuals dietary intake may alter the composition of the indigenous microbial flora, allowing an overpopulation of *Candida* species. The presence of high levels of carbohydrate in the gut may favour multiplication of yeast rather than bacteria (Cormane and Goslings, 1963) and carbohydrate-rich diets have been linked to increased oral *Candida* colonisation (Samaranayake, 1986). Several publications from the Far East suggest a higher prevalance of *Candida* in the malnourished (Gracey *et al.*, 1974; Sunoto *et al.*, 1980) and vitamin deficiencies may further predispose this sector to candidosis (Samaranayake, 1986).

(c) Mechanical Factors

Severely burned patients are particularly susceptible to colonisation and deep-seated infection with yeasts (Spebar and Blindberg, 1979) but the reported incidences of *Candida* colonisation varies from 11.4% (Spebar and Pruitt, 1981) and 54.7% (Law *et al.*, 1972). However. candidaemia can often occur in patients without infection of the burn itself (Spebar and Pruitt, 1981) suggesting other portals of entry or other factors, rather than the trauma itself, increases the likelihood of infection (MacMillan *et al.*, 1972).

The natural distribution of *Candida* species within the body suggests a preference of the yeasts for moist habitats, such as the groin and toe webs, rather than the general skin surface (Somerville, 1969). Therefore, any situation which involves covering or softening of the skin, or membranes, so as to raise local humidity may cause yeast overgrowth (Odds, 1988). The occlusive effects of nappies have been cited as a cause of *Candida* infection in babies (Dixon *et al.*, 1972), patients immobilised for long periods in hospital may show signs of *Candida* dermatitis (Yaffee, 1973) and higher oral yeast numbers are seen in denture wearers (Berdicevesky *et al.*, 1980) which may lead to yeast overgrowth between denture and palate.

(d) Iatrogenic Factors

The most cited factors which contribute to the development of candidosis arise from medical or surgical treatments. Their impact, however, is often difficult to evaluate because it is not easy to distinguish between their contribution and that of the underlying illness.

Antibacterial antibiotics which eliminate bacteria from human microbial habitats will allow yeasts to multiply more rapidly and their overgrowth leads to invasive infection (Seelig, 1966). Increases in *Candida* carriage have been attributed to tetracyclines (Mehta *et al.*, 1970), penicillin (Meads *et al.*, 1951) and ampicillin (Fitzpatrick and Topley, 1966) and a variety of studies have shown antibiotics to enhance colonisation and infection by *Candida* (Odds, 1988). There are no reports which demonstrate that antibiotics directly stimulate growth of *C.albicans* (Winner and Hurley, 1964). Treatment with corticosteroids, which produce pronounced anti-inflammatory and immunosuppressive effects, are very likely major factors in predisposing to superficial

(Gale, 1982) and systemic candidosis (Date *et al.*, 1983). The majority of the surveys find no statistical evidence for the effect of hormonal contraceptives on the prevalence of *Candida* in the vagina, although the oestrogen component of the hormonal contraceptive has a greater effect on *Candida* carriage than the progesterone component (Jackson and Spain, 1968).

With increasing advances being made in surgical techniques systemic candidosis is emerging as a clear hazard (Bernhardt *et al.*, 1972). *Candida* infections have been described as complications in bone marrow transplantation (Berkowitz *et al.*, 1985), renal transplantation (Gallis *et al.*, 1975), liver transplants (Wajszczuk *et al.*, 1985) and heart-lung transplants (Brooks *et al.*, 1985). The use of in-dwelling catheters, especially those used in central circulation and parenteral nutrition, are associated with a high risk of systemic candidosis. *Candida* isolates have accounted for anywhere between 2% and 67% of positive catheter cultures and *C.albicans* has been identified in 80% of cases (Odds, 1988). It is probably realistic to assume that exogenous and endogenous sources can lead to contamination of catheters, with the yeasts readily adhering to (Rotrosen *et al.*, 1983) and colonising the plastic surface (Locci *et al.*, 1981).

1.3.3 Pathogenicity and Virulence

If a fungus is to be perceived as potentially pathogenic and has the ability to compete successfully within a susceptible host, a number of steps are required to be completed to overcome specific and non-specific host defence mechanisms. The attributes of a successful pathogen, according to Richardson and Shankland (1991), include ;

(a) the ability to adhere to host surfaces.

(b) the ability to penetrate and gain access to target organs.

(c) tolerance of often extreme environmental conditions and the ability to multiply *in vivo*.

(d) inherent resistance to the selective pressure of host defence mechanisms.

(e) the ability to cause damage to host tissues.

A wide range of virulence properties are seen in *Candida spp.*, especially *C.albicans*, and are accounted for by genomic variability. Cutler (1991) attempts to explain this variability as a 'virulence-set hypothesis'. Virulence traits displayed by an organism belong to a set of genes and a given isolate will only express a finite number of traits

which together will constitute its virulence phenotype. Possession of a single trait of the set may not be sufficient for virulence to be expressed, and furthermore not all genes within the set may be necessary. However, each isolate must express a critical number of genes that act in concert to enable the organism to cause disease. Therefore, no single factor contributes solely to the success of *C.albicans* and other *Candida spp.* as pathogens, however, the formation of hyphae, enzyme production, toxin production and adhesion are potentially the most important attributes.

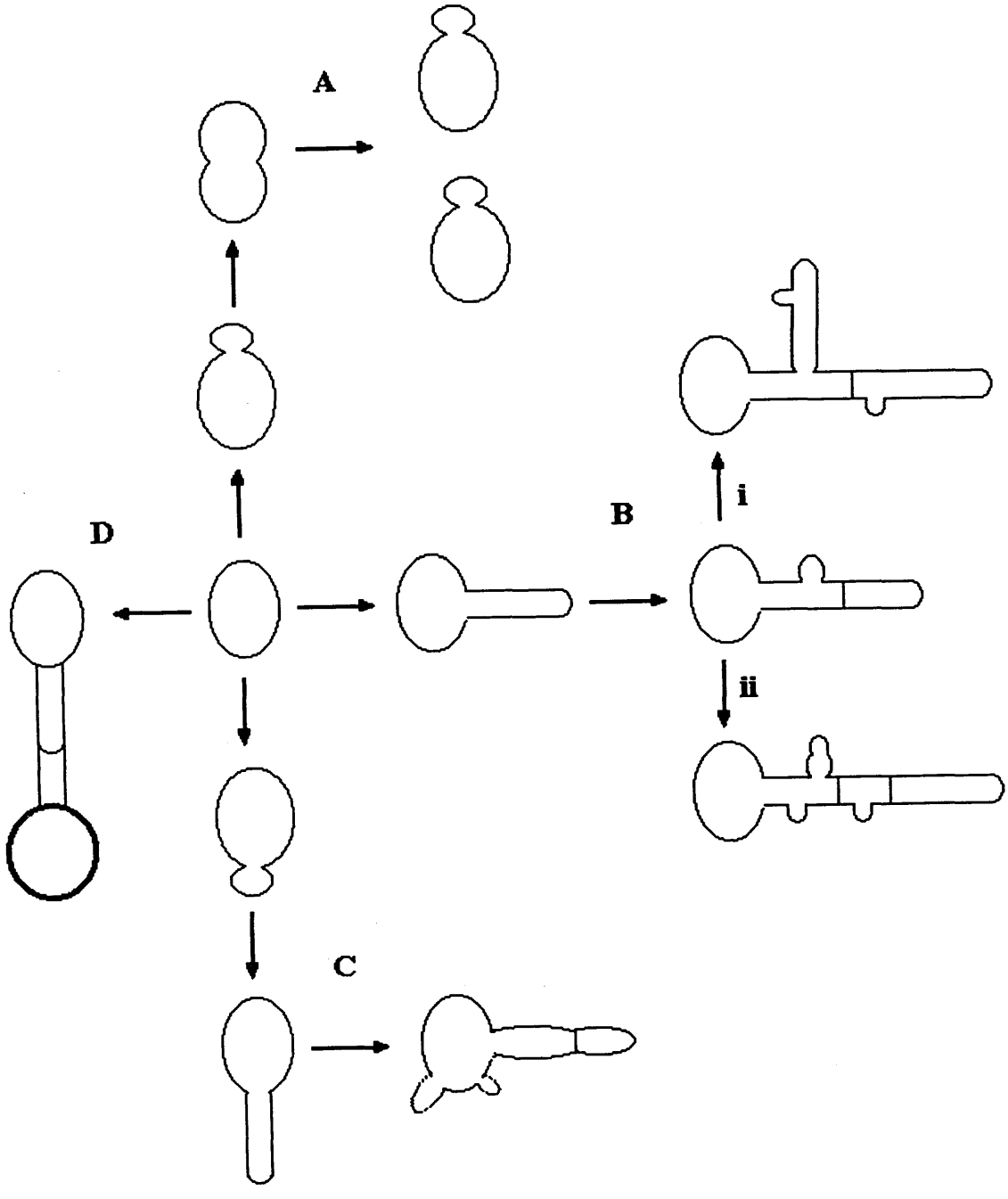
(a) Hyphal Production

C.albicans is a pleomorphic yeast since it can grow in one of at least four definable forms. A *blastospore* is the unicellular form of the fungus, a single yeast cell, that is distinguished by its specific process of cell division known as *budding* (Figure 1.4A). A parent blastospore produces a new cellular outgrowth from its surface from which a new bud, or daughter cell, develops, ultimately separating to form two blastospores. A *hypha* in *C.albicans* is a microscopic tube containing multiple fungal units separated by septa. Hyphae arise as branches from existing hyphae or blastospores in the shape of a *germ tube*. The hyphae give rise to lateral buds or hyphal branches (Figure 1.4B). *Pseudohyphae* closely resemble hyphae but differ in their formation: each new generation of buds remain attached to its parent and the progeny tend towards end-to-end aggregation of elongated buds (Figure 1.4C). *Chlamydospores* are large, refractile, thick-walled cells subtended from hyphae or pseudohyphae by specialised 'suspensor cells'. They are only formed, however, *in vitro* by *C.albicans* (Figure 1.4D) (Odds, 1988).

A great deal of speculation exists concerning the relationship between the virulence of *Candida* strains and the yeast to hyphal transition. It is presumed that hyphae are mechanically better suited than yeast cells for tissue penetration and invasion and may express molecular virulence factors which assist in the pathogenesis of candidosis. However, conflicting evidence is documented concerning the absolute requirement for the hyphal form of the fungus to initiate infection (Odds, 1988).

Shepherd (1985b) examined the relative pathogenicities of the yeast and mycelial forms of *C.albicans* after intravenous injection into mice, kidney sections from the infected mice showed the presence of both forms and pseudomycelium. Ryley and Ryley (1990)

Figure 1.4 - Schematic diagram showing the formation of different morphological forms in *C.albicans* (adapted from Odds, 1988).



A. Budding process in which new buds develop from the parent blastospore.

B. Hypha formation in which the germ tubes give rise to (i) hyphal branches, or (ii) laterally budding blastospores. Septa are laid down by the continuously extending hyphal tip.

C. Pseudohypha formation, a morphogenetic intermediate between budding and hyphal growth, where buds tend to elongate and not separate.

D. Chlamydospores developed at the tips of pseudohyphae, subtended from 'suspensor cells'.

using morphological mutants, growing solely as one or other form, showed that both morphologies had a propensity to cause disease in the murine model and concluded that hyphae were not in fact required to initiate disease. It was suggested by Sobel *et al.* (1984) that germination to the hyphal form was important in vaginal colonisation and pathogenesis of vaginitis, however, blastospores could also successfully colonise the vagina but produce a milder infection.

Hyphae are considered to be responsible for cell penetration and invasion and also have been found to adhere more successfully to epithelial cells than the yeast form (Kimura and Pearsall, 1978; Sobel *et al.*, 1984), but there is no existing evidence that one form can predominantly cause candidosis. Rather the heightened degree of pathogenicity of *C.albicans* may be a direct consequence of the interconversion between the two forms (Odds, 1988).

(b) Adhesion

Adhesion to host surfaces by pathogenic *Candida* species is both necessary for colonisation and as a prelude to tissue invasion (Douglas, 1987). The extent and strength of adhesion will depend on a number of parameters involving the fungus itself and macromolecules on its surface (adhesins), host surface receptors or environmental factors.

(i) Yeast Factors

Distinct differences are seen in the virulence of *Candida species, C.albicans* is undoubtedly the most virulent followed by *C.tropicalis* and *C.parapsilosis* (Douglas, 1992). These differences are closely parallelled by their ability to adhere to mammalian cells (King *et al.*, 1980) and other surfaces (Maisch and Calderone, 1980). Variation in adherence between strains is less obvious. *C.albicans* strains virulent for mice showed enhanced adherence, compared to commensal strains, only when grown in medium containing high concentrations of certain sugars. Under such conditions, these strains synthesise an adhesive fibrillar layer (Kearns *et al.*, 1983; McCourtie and Douglas, 1984). This phenotypic variation between commensal and invasive isolates is, therefore, likely to be an important virulence attribute and sugar-mediated control may be involved in establishment of oral infections where high concentrations of dietary sugars are present (Douglas, 1987).

Cell surface hydrophobicity (CSH) is a property of the *Candida* cell wall which may influence the progression of disease. Adherence and CSH expression are both temperature-dependant: cells grown at 25°C adhere better and are more hydrophobic than those grown at 37°C (Lee and King, 1983a; Hazen *et al.*, 1986) and morphological evidence of germination was preceded by the expression of CSH (Hazen and Hazen, 1988). It may be that surface hydrophobicity provides a mechanism which allows evasion of specific immune responses. Cells grown at 25°C were conjugated to and engulfed by polymorphonucleated neutrophils (PMNs) more than those grown at 37°C, but killed less often. Following engulfment germ tubes were produced more rapidly at 25°C affording a possible escape from the PMNs (Antley and Hazen, 1988). Preliminary studies suggest that surface proteins are involved in the expression of this hydrophobicity, and the conversion of hydrophilic to hydrophobic cells is due to changes in their mannosylation (Hazen *et al.*, 1990). Most of the experimental evidence currently available indicates a role for mannoprotein in yeast attachment to cells, and the predominant interaction is one involving the protein portion of the mannoprotein adhesin (Critchley and Douglas, 1987a). If the same molecule has hydrophobic characteristics the exposure of such a mannoprotein may represent an efficient mechanism for adhesion (Hazen *et al.*, 1990).

Klotz (1988) reports that a bioemulsifier enhances yeast adherence to human intestinal epithelial cells. A bioemulsifier produced by *Candida spp.* may change the surface characteristics of the target substrate to enhance adhesion and allow metabolism of the substrate, providing a nutritional advantage over non-adherent microorganisms (Gerson, 1985).

(ii) Host Epithelial Factors

Jones (1984) has described the adhesion process as a two component system which is dependant on the number and distribution of host surface receptors as well as adhesin production by the microorganism. A minimum number of adhesin-receptor interactions must be achieved for successful adhesion by an invasive fungus (Kennedy, 1988). This accounts for the different receptivities of epithelial cells from different sites (King *et al.*, 1980), from donor to donor, with age of donor (Cox, 1983) and the hormonal status of the individual. Vaginal epithelial cells from groups of women known to be predisposed

to vaginitis (pregnant and diabetic) bound more yeast than a control group, and cells from post-menopausal women bound least (Segal *et al.*, 1984).

(iii) Environmental Factors

Environmental temperature can affect the adherence of *C.albicans* to host surfaces by altering the surface properties or by affecting yeast cell germination (Kimura and Pearsall, 1980). Adherence is also affected by environmental pH (Ghannoum and Abu-Elteen, 1991). The optimal pH for adherence of both bacteria and fungi is reported to be in the range pH 6-8. However, when *C.albicans* was incubated with buccal epithelial cells at pH values ranging from 3-8, peak adherence by *C.albicans* was reported at pH 3 (Samaranayake and MacFarlane, 1982). This suggests that the effect of pH on adherence varies with the source of mucosal cells (Mehentee and Hay, 1989).

For significant adhesion and colonisation in the gastrointestinal tract the ecology of endogenous microflora must first be disrupted by antimicrobial treatment or chemotherapy (Kennedy, 1988; Macura, 1988) before *Candida* cells can proliferate, or the fungus must be implanted before mucosal flora is established (Field *et al.*, 1983). Preformed antibodies to *Candida spp.* may also inhibit adherence and subsequent pathogenesis (Scheld *et al.*, 1983).

(iv) Adhesins and Host Receptors

Ultrastructural studies suggest that an outer floccular-fibrillar layer is responsible for the interaction between the fungus and the host surface (Marrie and Costerton, 1981). Reports vary as to the physical dimensions of this layer (Lee and King, 1983b; Kennedy and Sandin, 1988) and it has been described as being unevenly distributed on the cell surface (McCourtie and Douglas, 1981), occasionally as having an ordered alignment around the cell (Tronchin *et al.*, 1984) or being localised at an adhesive site (Howlett and Squier, 1980).

A number of components of the yeast cell wall have been suggested as possible adhesins, and include mannan or mannoprotein (Sandin *et al.*, 1982; Douglas and McCourtie, 1983), chitin (Segal *et al.*, 1982) and evidence for the involvement of lipids has recently emerged (Ghannoum *et al.*, 1987).

A mannoprotein adhesin of *C.albicans* is thought to bind via its protein moeity to a glycoside on the surface of buccal epithelial cells (Douglas, 1991). Adherence of *C.albicans* can be inhibited by pretreatment of cells with Concanavilin A, which binds

to α-linked D-mannose residues (Sandin *et al.*, 1982), proteolytic enzymes (Sobel *et al.*, 1981) or reducing agents such as β-mercaptoethanol (Lee and King, 1983b). Tunicamycin, an antibiotic which specifically inhibits the glycosylation of mannoprotein in yeast, inhibits the formation of a fibrillar layer in a high galactose medium which results in reduced adherence (Douglas and McCourtie, 1983).

Most known receptors for microorganisms on the surface of animal cells are the carbohydrate components of the membrane bound glycoproteins or glycolipids (Jones and Isaacson, 1983). Critchley and Douglas (1987b) reported that glycosides containing L-fucose, N-acetyl-D-glucosamine and possibly D-mannose can function as epithelial receptors for different strains of *C.albicans*. Rotrosen *et al.* (1985) proposed that the host cell receptor for *C.albicans* was fibrinonectin, an important modulator of bacterial adhesion and colonisation in the mouth, which acts as a receptor for some species but not others (Simpson *et al.*, 1982). A study by Lancaster and Douglas (1986) demonstrated that fibrinonectin inhibited the attachment to epithelial cells of several *Candida* species, therefore, fibrinonectin may provide a 'barrier' to colonisation and infection. It is known that seriously ill hospital patients have a reduced level of fibrinonectin on their buccal cell surfaces (Douglas, 1987) which may predispose them to fungal infection.

(c) Enzyme Production

Hydrolytic enzyme activities which are expressed at the surface of microbial pathogens are always able, at least in theory, to cause damage to host cells *in vivo* (Odds,1988). Two enzymes with this potential in *C.albicans* have been described, a proteinase and a phospholipase, but their role as virulence factors remains ambiguous.

Staib (1965) first reported extracellular proteolytic activity in *C.albicans* strains which could utilise serum proteins as a source of nitrogen, and consequently related this to strain pathogenicity (Staib, 1969). MacDonald and Odds (1980b) suggested such a role for extracellular proteinase on the basis of the presence of the enzyme in tissue lesions, as determined by indirect immunofluorescence, and the presence of antibodies to the enzyme in patients with systemic candidosis (MacDonald and Odds, 1980a). However, the enzyme may aid propagation of the fungus in the host, rather than act directly as a virulence factor, (Homma *et al.*, 1992) by assisting invasion and degradation of skin or

mucosa (Negi *et al.*, 1984; Kobayashi *et al.*, 1989), attacking antibodies, granulocytes and macrophages (MacDonald and Odds, 1983; Ghannoum and Elteen, 1986) or supplying nutrients by digestion of proteins (MacDonald, 1984; Shimizu *et al.*, 1987). Phospholipases have been found in many fungi and it has been suggested that they play a role in pathogenicity (Barret-Bee *et al.*, 1985). Price and Cawson (1977) demonstrated phospholipase activity in *C.albicans* cell extracts and associated this activity with bud formation during growth. During invasion of chick chorioallantoic membranes, yeasts showing high levels of phospholipase activity appeared to clear a path for invasion and colonisation of tissues by hyphae. Very high levels of enzyme activity were associated with the hyphal tips (Pugh and Cawson, 1977). A correlation was found between phospholipase levels, pathogenicity and adherence to epithelial cells in several *C.albicans* strains. Non-pathogenic *S.cerevisiae* and *C.parapsilosis* showed both lower enzyme activity and adherence (Barret-Bee *et al.*, 1985).

1.4 Use of Antifungal Agents against *Candida* Infections

The repertoire of available antifungals for the treatment of candidosis is considerable, however, an "ideal" compound is not a reality. Most of those available are suitable only for topical application and those to treat systemic infections are few. In short, none is yet so effective, so safe or so convenient that it can be used without qualification (Odds, 1988).

The potential targets for the currently available antifungals include the cell wall, plasma membrane, endoplasmic reticulum and the nucleus (see Table 1.3 for summary).

(a) Antifungals Impairing Barrier Function of the Plasma Membrane

The polyene macrolide antibiotics are the natural products of filamentous bacteria, usually *Streptomyces* species, and include Amphotericin B and nystatin. The polyenes selectively interact with the ergosterol-containing plasma membrane of the fungus, changing its physical state and causing impairment of barrier function. This results in enhanced permeability to protons and leakage of K^+, Ca^{2+} and PO_4^{3-} (Vanden Bossche *et al.*, 1987). However, this alone does not appear to account for all aspects of polyene action and Medoff (1988) suggested the involvement of oxidative processes in the fungicidal activity of Amphotericin B.

Amphotericin B has been the "gold standard" for treatment of systemic or life-threatening *Candida* infections since its discovery (Gold *et al.*, 1956). Resistant strains in clinical material are very rare, and seem only to arise in species of *C.lusitaniae* which affects neutropenic patients (Athar and Winner, 1971). Applied topically it has been successful in the treatment of oral, genital and cutaneous candidosis, however, because of its poor absorbance from the gut it must be administered intravenously for the treatment of systemic infection. However, Amphotericin B has proved to be highly toxic producing kidney damage and uremia in patients, therefore, a firm diagnosis of systemic candidosis should be made before administration.

Nystatin was the first-ever described polyene (Hazen and Brown, 1950) and the first-ever specific antifungal agent to be used therapeutically for candidosis. It is a popular treatment for oral and vaginal forms but its toxicity and its insolubility have reduced its use to only topical treatments. No more than trivial side effects have, however, been reported (Odds, 1988).

Table 1.3 - Summary table of the potential targets for host-antifungal interactions (adapted from Vanden Bossche, 1991).

Antifungal Target	Localised Effect	Antifungal Agent
PLASMA MEMBRANE	Impairment of barrier function	**Polyenes:** Nystatin and Amphotericin B **Azoles:** Clotrimazole and Fluconazole
ENDOPLASMIC RETICULUM	Ergosterol biosynthesis	**Azoles:** Miconazole and Itraconazole
CELL WALL SUBUNITS	Chitin synthase	Polyoxin and Nikkomycin
	Chitinase enzyme	Allosamidin
	B-(1,3)-D-glucan synthase	Papulacandin, Cilofungin and Echinocandin
	Mannoprotein synthase	Tunicamycin
NUCLEUS	Inhibition of DNA and RNA synthesis	Flucytosine

(b) Antifungals Affecting Enzymes of the Endoplasmic Reticulum

An important group of modern antifungals, the azoles, interfere with the biosynthesis of ergosterol the main sterol component of fungal cell walls. The azoles can be considered in three broad groups with respect to their historical development (Odds, 1988);

(1) **1st generation azoles** are topically active imidazole derivatives and include clotrimazole and miconazole. In common with all azoles, they cause alterations in the composition of *Candida* membranes by their action on cytochrome P450 during ergosterol biosynthesis. At high concentrations they can also be fungicidal by directly damaging the cell membrane (Sud and Feingold, 1981).

(2) **2nd generation azoles** are orally active N-substituted imidazoles, exemplified by ketoconazole, but they do not have the direct membrane-damaging capabilities of the 1st generation azoles.

(3) **3rd generation azoles** are the triazole derivatives, which again are not direcly membrane damaging.

Differences in the extent of inhibition of fungal and mammalian cytochrome P450's accounts for the selective toxicity of azoles for fungi (Vanden Bossche *et al.*, 1984) and they are thought to bind to the haem portion of the fungal cytochrome via their imidazole or triazole moeity, thus blocking the site normally occupied by oxygen. At higher concentrations 1st generation azoles can damage membranes and induce changes in amino acid transport (Vanden Bossche, 1974), cause leakage of intracellular contents, reduction of cellular ATP (Ansehn and Nilsson, 1984) and inhibit DNA and RNA synthesis (Iwata *et al.*, 1973). The differential membrane-damaging effects of the azoles are probably due to the extent of the reorganisation of membrane lipids produced in each case (Vanden Bossche *et al.*, 1982). They can also prevent or perturb hyphal development (Odds *et al.*, 1985), therefore, since the hyphal form may be an important attribute to the pathogenicity of the fungus, the azoles may prevent development of invasive candidosis in the host.

With several of the azoles, clotrimazole and miconazole, there are problems associated with absorption from the gastrointestinal tract so they are mainly used therapeutically, like Amphotericin B, in the treatment of cutaneous *Candida* infections.

Ketoconazole was the first truly active azole antifungal (Heeres *et al.*, 1979) and given orally is suitably well absorbed to give rise to useful blood and tissue levels, unless patients are receiving antacids or H2 blockers (Van Tyle, 1984) when absorption is reduced. Ketoconazole is the drug of choice for chronic cutaneous candidosis (Graybill, 1989) and for the treatment of documented thrush (Van Tyle, 1984). However, several reports suggest the drug may be teratogenic (Fromtling, 1984) or cause male sterility and a few cases of hepatotoxicity have been reported (McNair *et al.*, 1981).

Fluconazole is an orally active triazole antifungal which has lower protein binding, slower clearance than ketoconazole and excellent penetration of tissues, including cerebrospinal fluid (Humphrey *et al.*, 1985; Arndt *et al.*, 1988) and a broad spectrum of activity (Graybill, 1989). Fluconazole is systemically active as an oral agent with proven activity against *Candida* infections in AIDS (Chave *et al.*, 1990), however, the drug has been increasingly associated with selection of azole-resistant species and strains of *Candida* (Kitchen *et al.*, 1991).

(c) Antifungals Interfering with Macromolecular Synthesis

Flucytosine was initially developed as an anti-cancer drug and of all the compounds that inhibit macromolecular synthesis in fungi it is the only one that has been exploited in the treatment of patients suffering from fungal disease (Vanden Bossche, 1991). Flucytosine is actively transported into sensitive yeast cells by a cytosine permease (Polak and Scholer, 1973) where it is converted to 5-fluorouracil by the enzyme cytosine deaminase. The latter compound is metabolised by the pyrimidine salvage pathway to 5-fluorouridine triphosphate (5-FUTP) and 5-fluordeoxyuridylate (5-FdUMP). 5-FUTP is incorporated into fungal RNA, with consequences for protein synthesis (Polak and Scholer, 1975) and 5-FdUMP is a potent inhibitor of thymidylate synthetase, a key enzyme in DNA synthesis (Diasio *et al.*, 1978). Selectivity arises from the fact that man lacks a cytosine deaminase to convert cytosine to uracil (Vanden Bossche, 1991).

The drug is less toxic than Amphotericin B with the most common, and less serious side-effects being nausea, diarrhoea and skin rashes, liver toxicity is very rare. Good oral absorption and this comparitive safety are, however, offset by the high prevalence of resistance among *Candida* species (Scholer, 1980). *C.tropicalis*, *C.parapsilosis* and

C.krusei seem to show a higher prevalence of resistant strains than *C.albicans* (Nobre *et al.*, 1981) and because of this flucytosine is used primarily in the treatment of systemic infections of the lungs, urinary tract, central nervous system and disseminated candidosis (Odds, 1988).

(d) Antifungals Interfering with the Synthesis or Hydrolysis of Cell Wall Subunits

Any impairment in the balance between the synthesis of new cell wall components and the controlled and localised hydrolysis of pre-existing polysaccharides will lead to an imbalance in growth, bud formation and septation. Chitin, mannan and several glucans of the *Candida* cell wall are not found naturally in the human host, so they can, in theory, be targeted without toxicity to the host (Vanden Bossche, 1991). Thus, the cell wall would appear to be an ideal target for antimycotic action and is the current focus of several new antifungals.

Polyoxins, analogues of UDP-N-acetyl-D-glucosamine produced naturally from *Streptomyces cacoi* var. *asoenis* (Ryley *et al.*, 1981), inhibit chitin synthase in cell-free systems (Emmer *et al.*, 1985). These compounds cause a reduction in septal fluorescence with chitin-specific stains (Hilenski *et al.*, 1986) and other morphological effects (Shenbagamurthi *et al.*, 1983) at high concentrations. However, most polyoxins are rapidly degraded by whole cells of *Candida*. Similarly, the nikkomycins show strong chitin synthesis inhibition *in vitro* (Hector and Braun, 1986) and the tunicamycins, a third group of the UDP-N-acetyl-D-glucosamine analogues, prevent glycosylation of cell wall proteins. They have been shown to prevent incorporation of mannoproteins into regenerating sphaeroplasts of *C.albicans* (Murgui *et al.*, 1986) but like polyoxins are less effective against whole cells.

Chitinase inhibitors may have an advantage over chitin synthase inhibitors as a target because chitinase may be accessible without requiring transport across the membrane (Vanden Bossche, 1991). Allosamidin, a *Streptomycete* antibiotic, was reported to inhibit insect chitinase (Koga *et al.*, 1987) and the chitinase of *Neurospora crassa* (Vanden Bossche , 1991), so it may be that it also has the potential to inhibit *C.albicans* and other pathogenic fungi.

A further potential target is the plasma membrane associated synthesis of glucans. The papulacandins, metabolites of *Papularia sphaerosperma*, are glycolipids which inhibit

β-(1,3)-D-glucan synthetase in yeasts, thus, weakening cell wall structure (Rommele *et al.*, 1983). Two related antifungal antibiotics are the echinocandins, produced by various *Aspergillus* species, and aculeacin A. They are both cyclic-peptides which inhibit glucan synthesis resulting in cell wall deformation and cell lysis (Cassone *et al.*, 1981; Yamaguchi *et al.*, 1982). Cilofungin is an echinocandin derivative which competively inhibits β-(1,3)-D-glucan synthase activity of *C.albicans* isolates and is at least 20-fold less toxic than Amphotericin B (Gordee *et al.*, 1988), however, other *Candida* species such as *C.glabrata* and *C.parapsilosis* are less susceptible to its action (Hall *et al.*, 1988). Thus, the spectrum of activity of the antifungals affecting glucan synthesis may be too narrow for treatment of mycoses other than candidosis due to *C.albicans.*

1.4.1 Antifungal Prophylaxis

Preventative measures against life-threatening invasive mycoses requires serious consideration in order to halt further increases in the numbers of patients dying as a direct result of fungal infection. However, the direct benefits of chemoprophylaxis are controversial (Meunier, 1992) and the use of antibacterials and antifungals to prevent the overgrowth of commensal microbes is a complex and controversial area (Odds, 1988). No drug or combination of drugs have been successful in the total eradication of yeast microflora. However, several studies with Amphotericin B (Guiot *et al.*, 1981), nystatin (Carpentieri *et al.*, 1978), ketoconazole (Meunier *et al.*, 1989) and fluconazole (Samonis *et al.*, 1990) have reported a reduction in yeast carriage and risk of disseminated candidosis.

Perhaps total eradication of the pathogen is unrealistic and interference with virulence mechanisms, such as the morphological switch (Bennett, 1984) or adherence to host cells (Sobel and Obedeanu, 1983), will be sufficient to reduce the risk of serious infection.

As an alternative to specific antifungal agents, other potentially effective measures should be considered. These include the education of patients and their families, further training of medical staff and the use of surveillance cultures (Pfaller *et al.*, 1987) to aid bedside diagnosis and management (Meunier, 1992).

1.4.2 Identification of New Antifungal Targets

The identification and exploitation of new antifungal targets will undoubtedly rely on the ability to apply recombinant-genetic techniques to aspects of fungal cytology, metabolism and gene expression which are important in fungal pathogenesis but have no mammalian counterpart (Tuite, 1992). The virulence factors (section 1.3.3) of *C.albicans*, the most pervasive fungal commensal, warrant such attention.

Strong evidence exists for the involvement of a secreted aspartic protease in fungal pathogenesis and its presence in the human host has been detected from an early stage (MacDonald and Odds, 1980a). Recent identification of the aspartic proteinase-encoding gene from *C.albicans* (Hube *et al.*, 1991) and *C.tropicalis* (Togni *et al.*, 1991) has provided important information concerning the protease structure. This knowledge may facilitate the rational design of protease inhibitors.

Fungal-cell-wall biosynthesis is an attractive target for antifungal chemotherapy since the mammalian host cell presumably has no analogous pathways. The fungal cell wall is not an inert structure but one in continuous flux, undergoing profound changes as a consequence of budding, apical extension or hyphal development. Therefore, there are many functions which are possible points of interference as a result of the involvement of synthetases and hydrolases in cellular processes (Nombela *et al.*, 1991). Protein kinase involvement in cell-wall biogenesis has been identified in *S.cerevisiae* (Torres *et al.*, 1991) and it is feasible that it may act in a similar role in *C.albicans*. Although the kinases may not be directly targeted themselves, the proteins they phosphorylate may provide an alternative site of action for antifungals (Tuite, 1992).

Other aspects of the fungal cell wall also demand further attention, in particular the morphogenic switch in *C.albicans* and its ability to adhere to host cells. A diverse collection of signals (pH, temperature and nutritional factors) can induce the yeast-hyphal transition, yet these signals do not appear to share any common properties. The receipt and transmission of these signals to their intracellular targets may involve a second-messenger system. A role for the involvement of calcium and calmodulin in morphogenesis has been suggested (Sabie and Gadd, 1989) and Paranjape *et al.* (1990) found that calmodulin activity increased during hyphal development. However, the level of transcription of the calmodulin gene does not appear to vary between the two forms (Saporito and Sypherd, 1991) so control may lie at the post-translational level (Tuite,

1992). The nature of the *C.albicans* adhesins remains undefined, but elucidation of their structure may provide an opportunity to develop an antifungal which inhibits the host-pathogen interaction.

Identification of a potential target, however, is only the first step in producing an effective antifungal agent. A high throughput screen must then be developed to identify potential inhibitors which act on the target, and the structure of the latter must be well-defined to facilitate rational drug design (Marriot, 1990).

1.5 Aims and Objectives

Initially the project was planned to involve the screening of a number of putative killer yeasts, using traditional agar diffusion bioassays, to identify a strain with strong antimicrobial activity against pathogenic strains of *Candida*. Subsequently the objectives were to produce the extracellular killer factor in a series of fermentations for an investigation into the pharmacology of the selected killer yeast strain. Studies were to involve;

(a) the determination of the spectrum of action of the killer yeast against clinical isolates of *Candida*,

(b) the determination of the biochemical and physico-chemical activity of the killer factor produced, and

(c) the elucidation of the possible mode of action of the killer toxin against sensitive yeast strains.

Purification of the killer factor to homogeneity using chromatographic techniques would potentially allow the bioactive peptide to be determined. It was hoped that this information would contribute to the understanding and synthesis of a novel antifungal with the potential for therapy of *Candida* infections.

CHAPTER TWO

Materials and Methods

2.1 Culture Collection and Maintenance

Yeast strains held at the Institute were obtained from the National Collection of Yeast Cultures (NCYC, England), the American Type Culture Collection (ATCC, U.S.A) and the Centraalbureau voor Schimmelcultures (CBS, Netherlands). *W.mrakii* LKB-169, was kindly donated by Dr. Kodama (Laboratory of Kodama Brewing Co. Ltd., Japan) and *S.cerevisiae* 28, by Dr. Pfeiffer (Institut fur Mikrobiologie und Weinforschung, Germany). Clinical isolates of *Candida* were obtained from Glaxo Group Research (Middlesex, England), Ninewells Hospital (Dundee, Scotland) and Trinity College (Dublin, Ireland).

All yeasts were inoculated and subcultured monthly on Sabouraud Dextrose Agar (SDA, Oxoid, Unipath Ltd., Basingstoke, UK) slopes at 25^{O}C for 48 hours and then maintained at 4^{O}C until required.

2.2 Identification of Clinical Isolates

Microring YT (Medical Wire and Equipment Co. (Bath) Ltd., Wiltshire, England) was an *in vitro* diagnostic system used to identify the isolates obtained from Ninewells Hospital. Each Microring device carried six tips which were impregnated with a range of antimicrobial agents and dyes, each tip performed as an individual disc test because of an isolating hydrophobic barrier. The components of each tip were; **1** Janus green (156 µg); **2** Ethidium bromide (37 µg); **3** Triphenyl tetrazolium bromide TTC (150 µg); **4** Brilliant green (200 µg); **5** Cyloheximide (45 µg) and **6** Rhodamine G (60 µg).

A single colony of each isolate was removed using a sterile swab (BDH Laboratory Supplies, Merck Ltd., Lutterworth, UK) and plated on fresh SDA. Using sterile forceps, a Microring was placed in the centre of the plate and each tip pressed firmly to ensure contact with the surface of the agar. The plates were incubated at 37^{O}C for 24 hours and the response and zone sizes recorded. After an additional incubation for 24 hours, the presence and absence of brown or red colouration around the TTC tip was noted. The results allowed a six-digit code to be assigned to each isolate and these were compared to the profiles of known yeasts.

2.3 Determination of Cell Numbers

Yeast cell numbers were estimated using an Improved Neubauer haemocytometer (Weber, England) or an Industrial D Coulter counter (Coulter Electronics Ltd., Luton), for which the cells were suspended in Isoton II (Coulter Electronics Ltd., Luton), an osmotically balanced electrolyte solution, and passed through an aperture of 100 μm diameter for counting. To avoid clumping and, therefore, underestimation of cell numbers, solutions were sonicated (Transsonic T310 bath, Camlab) for 2 minutes prior to counting.

2.4 Estimation of Protein Content

Pierce Protein Assay Reagent (Pierce Chemical Company, USA), a ready-to-use Coomassie blue G-250 reagent solution, was used for the quantitative determination of protein concentration. The micro assay procedure was followed which determined protein concentrations in the range of 1-25 μg/ml.

A known protein concentration series was prepared by diluting a stock (2.0 mg/ml) bovine serum albumin standard (BSA, Pierce Chemical Company, USA) in distilled water. The protein standard series covered the range of concentrations between 1 and 25 μg/ml. One ml of standard protein, or toxin-containing sample, was pipetted into a test tube and 1.0 ml of Coomassie Assay Reagent was added. The solutions were mixed well and after 2 minutes the absorbance of the solution at 595 nm was measured using a Novaspec II spectrophotometer (Pharmacia LKB Biochrom Ltd., Milton Keynes, UK), against a distilled water blank. A standard curve was prepared from the values for each BSA standard and this was used to determine the protein concentration of each unknown toxin sample.

2.5 Estimation of Carbohydrate Content

The colourimetric phenol-sulphuric acid assay of Dubois *et al.* (1956) was followed. A known glucose concentration series was prepared in distilled water. The glucose series covered the range of concentrations between 5 and 300 μg/ml. Two ml of the sugar solution (standard or sample) was pipetted into a test tube and 1 ml of 5% phenol (BDH) in water was added. Then 5 ml of concentrated H_2SO_4 (BDH) was added rapidly, the stream of acid being directed at the liquid surface rather than against the side of the test

tube in order to obtain good mixing. The tubes were allowed to stand for 10 minutes then were shaken and placed in a water bath at 25-30^{o}C for 15 minutes. the absorbance of the solutions was read at 490 nm against a distilled water blank. The amount of carbohydrate present was then determined by reference to a standard curve previously constructed. All solutions were prepared in triplicate to minimise errors.

2.6 Assessment of Killer Toxin Activity

2.6.1 Streak-Plate Agar Diffusion Bioassay

A modification of the streak-plate assay of Stumm *et al.* (1977) was developed to screen for killer yeast activity. Methylene blue agar (MBA) was prepared in citrate-phosphate buffer, pH 4.5 by the addition of 2% bacteriological agar (Oxoid), 2% Sabouraud Liquid Media (SLM, Oxoid) and 1% tryptone (Oxoid). They were heated to 100^{o}C prior to the addition of 0.003% methylene blue (BDH) and 5% glycerol (Aldrich). After autoclaving, (121^{o}C, 15 psi, 15 minutes) 15 ml aliquots were cooled to 45^{o}C before addition of the sensitive yeast at a density of $5x10^{4}$ cells/ml. The cells were mixed gently with the agar to prevent formation of air bubbles and poured into plastic 90 mm x 15 mm Petri dishes (Sterilin). A single colony of the presumptive killer was streaked on the agar surface using a flamed metal loop. Plates were incubated at 25^{o}C for 2-3 days. The sensitive strain grew as a background lawn and killer activity was evident as a zone of clearing surrounding the streak, which was marked by dead blue-stained colonies. Plates were scored using a visual system as outlined below:

- \- No killer activity
- * Trace activity
- ** Weak activity
- *** Moderate activity
- **** Good activity
- ***** Excellent activity
- (+) Undefined zone of clearing
- 0 Absence of blue-stained colonies
- 1 Staining beneath streak only
- 2 Distinct border of stained colonies

2.6.2 Agar Diffusion Well Bioassay

To assay crude toxin preparations, wells were cut in the seeded agar using a sterile well-borer (5 mm diameter) and removed with a sterile scalpel. Samples of cell-free supernatants (30-100 µl) were pipetted into the wells and the plates were incubated at $_{25}$oC for 2-3 days. Subsequent zones of inhibition produced around the wells were measured to the nearest 1.0 mm. The killing activity of each sample was measured as the mean zone of inhibition of five replicate wells in two plates. Plate 3.2 (page 56) shows the typical zones of inhibition produced.

2.6.3 Microtitre Plate Assay

Aliquots (180 µl) of SLM, seeded with $1x10^5$ cells/ml of the sensitive strain, were pipetted into microtitre wells (Nunc MicroWell Modules, Denmark) and 20 µl of control solution, or the appropriate dilution of toxin, was added and mixed using the pipette tip. The wells were incubated at 30^{o}C for 18-20 hours and the absorbance of each was measured at 450 nm using an automatic plate reader (Titertek Multi-Scanner, Flow Laboratories, Irvine). Toxin activity was expressed as the percentage reduction in growth of the sensitive with respect to a toxin-free control. Where possible, samples were assayed in duplicate and compared to the mean absorbance of at least five control incubations.

At toxin levels below the 'threshold limit' of the assay, the ratio of toxin sample : seeded media was increased to 100 : 100 µl so that lower levels of toxin activity could be detected.

2.6.4 MTT-Colourimetric Assay

A 10% inoculum from an overnight culture of *C.glabrata* (S-388) was transfered to fresh SLM and incubated, with shaking, at 30^{o}C until late log-phase was reached. One ml samples containing approximately $1x10^8$ cells/ml were aliquoted into Eppendorf tubes to which was added 250 µl of toxin solution, or toxin-free Yeast Nitrogen Base-Glucose-Salts (YNBGS). The suspensions were incubated at room temperature for 90 minutes. The cells (500 µl) were mixed with 50 µl of 5 mg/ml [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT Sigma Chemical Company, Ltd., UK) and incubated at 30^{o}C for 2 hours. The suspension was then mixed with 500 µl of

isopropanol, (BDH) containing 0.04 M HCl (BDH). The mixture was vigorously vortexed (Whirlimixa, Fisons Scientific Equipment, Leicestershire, UK) to remove MTT-Formazan from the cells and then centrifuged at 12,000 rpm for 2 minutes. The absorbance of the supernatants at 570 nm was measured against a cell-free control solution using a Novaspec II spectrophotometer.

The results were calculated as:

$$\% \text{ viability measured by MTT assay} = \frac{OD_{Control} - OD_{Toxin}}{OD_{Control}} \times 100\ \%$$

The viability of the sensitive cells was also determined by colony forming activity on SDA after a 10^6 dilution of cell suspensions.

2.6.5 Membrane Damage Assay

The sensitive strain was grown overnight at 30°C in 50 ml of Glucose Nutrient Broth (GNB), comprising 2% glucose (BDH) and 1.3% nutrient broth (Oxoid). A 3 ml sample of cells was transfered to 50 ml fresh GNB and incubated, with shaking, at 30°C until mid-log phase was reached. Cells were harvested by centrifugation (IEC Centra-4B Centrifuge, International Equipment Company, USA) at 4,000 rpm for 2 minutes and washed with 10 ml buffer S comprising 100 mM sodium phosphate (Fisons) and 2% glucose, pH 6.0. The pellet was resuspended in 5 ml buffer S and incubated at 30°C for 30 minutes, 100 µl of 2-amino[^{14}C]isobutyric acid (Sigma) was then added and the cells were incubated for a further 30 minutes. The cells were harvested (4,000 rpm for 1 minute), washed twice with buffer S containing 5 mg/ml sodium azide (BDH) and resuspended in 5 ml of the same buffer. The cell suspension (25 µl) was added to microtitre wells with 100 µl of toxin, or control solution. The plate was incubated for 60 minutes at 30°C in a Dynatech shaker (Dynatech Laboratories Ltd., Billingshurst, UK) before cells were harvested onto filtermats (Skatron Instruments Ltd., Newmarket, Suffolk), using a Skatron harvester (Model 11055 Micro96), and were counted on a betaplate 1205 scintillation counter (LKB Scintillation Counter).

The results were calculated as :

$$\% \text{ Membrane Damage} = 100 - \frac{\text{Sample Count}}{\text{Control Count}} \times 100\ \%$$

2.7 *W.mrakii* (K-500) Killer Toxin Production

2.7.1 Static Culture Conditions

The rate of killer toxin production was compared in three media types:

(a) complex medium - comprising 0.3% SLM prepared in citrate-phosphate buffer, pH 4.5. The medium was autoclaved at 121^{o}C for 15 minutes.

(b) semi-synthetic medium - YEPD, comprising 1% yeast extract (Oxoid), 2% mycological peptone (Oxoid) and 2% glucose was made up in distilled water and autoclaved at 110^{o}C for 20 minutes (the reduced temperature was used to prevent glucose caramelisation).

(c) minimal medium - YNBGS, comprising 0.67% Yeast Nitrogen Base with amino acids (Difco Laboratories, Detroit, Michigan, USA), 2% glucose, 0.5% $(NH_4)_2SO_4$ (BDH) and 0.25% $MgSO_4.7H_2O$ (Fisons). YNB and glucose were prepared as a (x10) strength solution and filter sterilised through a 0.45 µm cellulose acetate membrane (Whatman Ltd., Maidstone, UK). The 'salts' were autoclaved and the glucose and YNB added aseptically.

Conical flasks containing 50 ml of the appropriate media were inoculated with *W.mrakii* and incubated under static conditions at 25^{o}C. Samples were removed and centrifuged at 13,000 rpm for 5 minutes (MSE, Micro Centaur) to produce cell-free supernatants, crude toxin preparation.

2.7.2 Small-Scale Fermentations

Overnight seed cultures (100ml) of *W.mrakii* were grown in YNBGS in 250 ml conical flasks at 25^{o}C. A 10% inoculum was used to initiate fermentations in one litre

(Gallenkamp Modular Fermenter, London, UK) or two litre (Electrolab Ltd., Tewkesbury, UK) fermenters. The pH and dissolved oxygen were monitored throughout the fermentation, if required, and the temperature was maintained at 25^{o}C. Samples were removed to estimate cell numbers, protein content and to determine the activity of the killer factor produced.

During exponential growth the mean generation time, was calculated from the following equation:

$$mgt = \frac{\log_{10} 2 \times t}{\log_{10} N - \log_{10} N_0} \quad (\text{hours}^{-1})$$

t = time interval (hours)

N_0 = starting cell population (cell numbers per ml)

N = final cell population (cell numbers per ml)

2.7.2.1 Effect of Agitation and Aeration on Toxin Production

Seed cultures of *W.mrakii* (K-500) were prepared by resuspending a slope culture in 1 ml of YNBGS to inoculate 100 ml of the same media. These were grown for 3 days at 25^{o}C prior to inoculation of each fermenter. Anaerobiosis was achieved in fermenter A by bubbling nitrogen gas through the fermenter for 1 hour, resulting in '0%'dissolved oxygen. Fermenter B was continuously aerated at a rate of 200 cm^3/min to produce a system with '100%'dissolved oxygen. A third fermenter C was gently agitated prior to, and after, inoculation to produce low residual levels of oxygen. The killer yeast was cultured for 161 hours at a temperature of 25^{o}C.

2.7.3 Large-Scale Fermentations

Preliminary inoculum development involved the transfer of a colony of the killer yeast *W.mrakii* (K-500) to YNBGS media (50 ml), which was incubated overnight at 25^{o}C under static conditions. Samples (10 ml) were transfered to eight 500 ml flasks containing 250 ml of the same media. The flasks were incubated for eight hours at 25^{o}C. The two litre starting inoculum was added to a 70 litre Biolafitte fermenter

containing 40 litres of YNBGS. The running conditions of the fermentation were as follows;

(a) temperature - 25^{o}C

(b) pressure - 0.5 bar

(c) aeration - 3.5 litre/min

(d) agitation - 150 rpm

Silicone 520 antifoam was also added to the fermenter.

During the fermentation, samples were removed to monitor possible contamination, cell growth, protein levels and toxin production. After 48 hours, the fermentation broth was processed as outlined below.

2.8 Downstream Processing of *W.mrakii* (K-500) Killer Toxin

2.8.1 Small-Scale Processing

The culture broth of the killer strain K-500 was centrifuged at 4,500 rpm for 10 minutes to produce a cell-free supernatant. The crude toxin was then microfiltered through 0.45 µm cellulose acetate membranes to further clarify the preparation. During the early stages of the project, Amicon stirred ultrafiltration units (Amicon Corp., Lexington, Massachusetts, USA) were used to concentrate the extracellular medium. A PM10 membrane (Diaflo, Ultrafiltration Membranes, Amicon Corp., Scientific Systems Division, USA) was washed, to remove the glycerol coating, and inserted into the cell. Nitrogen gas (40 psi) was passed into the unit, which was continuously stirred, and 50 ml of the supernatant was reduced to 10 ml (retentate). The concentrated sample was then freeze-dried (Micromodulyo 1.5K freeze-dryer, Edwards High Vacuum International, England) and the lyophilisate reconstituted in a reduced volume of distilled water. Permeate samples were also collected for further analysis.

2.8.2 Large-Scale Processing

A similar procedure was followed for the processing of large-scale fermentation products. 40 litres of fermentation broth was continuously centrifuged (Sharples A516PY, Sharples Scientific Ltd., Coventry, UK) at 15,000 rpm to remove all cellular material. The supernatant was further clarified by microfiltration through 0.45 µm

cassettes (20 ft^2) in a Millipore Pelicon unit. The supernatant was concentrated by Millipore Pelicon ultrafiltration (10 kDa cut-off) to 3.5 litres. The retentate was then freeze-dried using an in-house designed and built system (Glaxo Group Research, Greenford).

2.8.2.1 Dialysis of Freeze-Dried Material

The dialysis tubing (Medicell International Ltd., London, UK) was submerged in a solution of 2% sodium bicarbonate (BDH) and 0.05% EDTA (BDH) and boiled for 10 minutes. The solution was discarded and the tubing boiled for a further 10 minutes in distilled water. This was repeated, and prior to use the inner and outer surfaces of the tubing were washed with distilled water. One end was sealed with a double knot and 5 ml of toxin preparation added. The air was expelled from the bag and the top end sealed. The bag was placed in 500 ml distilled water and agitated gently for 19 hours at $4^{o}C$. On removal, the bag was washed with 10 ml distilled water and the washings, along with the dialysate and dialysing solution, were assayed for toxin activity using a microtitre assay (section 2.6.3). The carbohydrate content of each solution was also determined (section 2.5).

2.9 Characterisation of *W.mrakii* (K-500) Killer Toxin

2.9.1 Temperature Stability of *W.mrakii* Killer Toxin

Crude toxin was concentrated 20-fold by freeze-drying cell-free supernatants and reconstituting the lyophilisate in distilled water. Samples of the killer toxin were incubated at a range of temperatures; 4, 18, 25, 30, 37, 50, 70 and $100^{o}C$. At specific intervals, dependant on the incubation temperature, aliqouts (100 µl) were removed and assayed for toxin activity against *C.glabrata* (S-388) using a microtitre plate assay.

2.9.2 Optimum pH of Killing Activity

The effect of pH variation on the killing action of *W.mrakii* killer toxin in agar diffusion bioassays was investigated. Crude toxin from a 72 hour static fermentation was added to wells in methylene blue agar plates seeded with the sensitive strains *C.glabrata* (S-388) and *C.albicans* (S-214392), see section 2.5.2. The plates were buffered over a range of

pH 3.5 to 8.0. Following an incubation at 25^{o}C for 2-3 days, the zones of inhibition produced by the killer factor were measured.

2.9.3 pH Stability of *W.mrakii* Killer Toxin

Concentrated toxin was diluted in 0.1 M citrate-phosphate buffer at a range of pH's and the resultant pH of the solutions was measured using a micro-electrode (BDH Glass + electrode). The solutions were incubated at 18^{o}C for a period of 18 hours and the toxin activity remaining was measured against *C.glabrata* (S-388), using a microtitre assay. The percentage reduction in growth of the indicator strain was assessed in each case, with respect to toxin-free buffer controls of equivalent pH.

2.9.4 Effect of Proteolytic Enzymes on Toxin Activity

Pepsin (pepsin A from porcine stomach mucosa, Sigma), pronase (from *Streptomyces griseus*, Fluka BioChemika) and trypsin (Type I from bovine pancreas, Sigma) were standardised using haemoglobin (bovine, Sigma) as a substrate. To assay trypsin and pepsin the enzymes were dissolved in distilled water, pronase was dissolved in 5 mM $CaCl_2$ (Fisons) and papain in 15 mM L-cysteine (Sigma), 6 mM EDTA (BDH). To 1 ml of enzyme, over a range of concentrations, 1 ml of 0.1 M sodium citrate buffer, pH 4.2 and 1 ml of 2.5% (w/v) haemoglobin were added. After incubation for 18 hours at 20^{o}C, 5 ml of 4.0% (w/v) trichloroacetic acid (TCA, Sigma) was added. The solutions were centrifuged (5,000 x g for 10 minutes) to remove TCA-insoluble material and the supernatants passed through 0.2 µm syringe filters (Whatman). The absorbance of the filtrates were measured at 280 nm, which gave an indication of the extent of hydrolysis produced by each enzyme. The concentration of enzymes which hydrolysed the haemoglobin substrate to the same extent were used to further characterise the killer toxin.

One ml of crude toxin was incubated with 0.5 ml of the enzyme solution for 18 hours at 20^{o}C and residual toxin activity was measured against *C.glabrata* (S-388) using a microtitre assay. The effects of native and denatured enzymes (autoclaved for 15 minutes) were also investigated and compared to the effects of control solutions of distilled water (pepsin) and 5 mM $CaCl_2$ (pronase).

2.9.5 PhastSystem Analysis of *W.mrakii* (K-500) Killer Toxin

(a) PhastSystem Electrophoresis

The gels (Pharmacia, 10-15% gradient gel) were placed on the separation bed on a volume (100 µl) of ultrapure water to create a good electrical contact. On top of the gels was placed the buffer strip holder and the electrode assembly. An equal volume of boiling mix (see Appendix 9.1) was added to the solutions and they were heated to 100^{o}C for 3 minutes. The toxin samples and molecular weight markers (Low Molecular Weight SDS 7-Sigma) were cooled, centrifuged at 12,000 rpm and applied using the PhastSystem applicators which applied 2 µl to the gel. The small, comb-like pieces have a series of wells which take up samples by capillary action. The samples are held until they are applied to the gel at a programmed time (see Appendix 9.1). The comb was positioned at the base of the gel *i.e.* at the cathode end. The gels were run electrophoretically for 20-45 minutes, depending on the concentraion of the samples. The gels were removed and placed in the separate developmental unit where they were silver stained, destained and preserved according to a preset method (see Appendix 9.1). This procedure took approximately 40 minutes and the gels were dried on a hot-plate.

(b) PhastSystem Isoelectric Focussing

The PhastGel IEF 3-9 (Pharmacia) was placed on the separation bed as before. With this procedure no buffer strips were required and the positions of the electrodes were altered accordingly. After prefocussing to form the pH gradient across the gel, the toxin samples and calibration markers (Pharmacia IEF 3-9) were applied to the centre, as previously described. After focussing (see Appendix 9.1 for program) the gels were removed and silver stained as described above.

2.10 Purification of *W.mrakii* (K-500) Killer Toxin

2.10.1 Analysis using Fast Protein Liquid Chromatography (FPLC)

A Mono Q HR 5/5 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was washed and equilibriated at a flow rate of 1.5 ml/min with two buffers A and B (buffer B = buffer A + 1M NaCl) using a programmed procedure (see Appendix 9.2). Concentrated crude toxin was filter sterilised through a 0.22 µm pore-size syringe filter and 500 µl applied directly to the column. The material was eluted at a flow rate of 1.0

ml/min using a linear gradient of buffered NaCl (0-30% buffer B), strongly bound protein was removed using 100% buffer B (see Appendix 9.2 for complete elution programme). The eluate from the column was monitored by a single path UV monitor (Pharmacia UV-1) and chart recorder (LKB Bromma 2210 1-channel recorder) and 1 ml samples were collected (Pharmacia Frac-100) and assayed for activity. Elution profiles were obtained for different buffer systems over the range of pH 4.0 - 8.0.

2.10.2 Analysis using Gel Filtration Chromatography

Three column sizes were used for the analysis of killer toxin;

(a) commercial pre-packed Pharmacia Sephadex G-25M column (bed volume = 9ml)

(b) small, prepared Sephadex G-25 column (bed volume = 70ml)

(c) large, prepared Sephadex G-25 column (bed volume = 500ml)

For columns b and c the Sephadex powder (Pharmacia) was mixed with excess distilled water and allowed to stand at room temperature overnight. The swollen gel was degassed under vacuum to remove any dissolved air and the slurry was poured directly into the vertically mounted column using a glass rod. The space above the gel was filled with distilled water and the top-piece inserted.

Flow-rates of the columns were determined by measurement of the through-flow of water over a fixed time period. The rate of the pump (Eyela Microtube Pump MP-3, Tokyo Rikakikia Co., Ltd.) was adjusted accordingly and the fraction collector (LKB Bromma 2112 Redirac) programmed to collect appropriate sample volumes. Distilled water was pumped through for 1 hour to wash and equilibriate the columns. The homogeneity of the bed was checked by observing the progress of a Blue dextran-chromate mix (25 µg/ml). Blue dextran was used to determine the void volume of the column since it is excluded from the matrix of the gel by virtue of its high molecular weight (2 x 10^6 Daltons), and potassium dichromate to determine the exclusion volume. Freeze-dried material from a large-scale fermentation (see 2.7.3) was used for initial studies by gel filtration chromatography. A specific amount of material was reconstituted in distilled water, filter sterilised and applied directly to the column. Each column was eluted with distilled water and fractions were collected to assay for activity

using a microtitre assay. The absorbance at 280 nm of each fraction was measured (Pharmacia LKB-Ultrospec III) to monitor the protein profiles of each separation.

2.10.2.1 Adsorption of *W.mrakii* Killer Toxin to Sephadex G-25

Concentrated (20-fold) cell-free supernatant was diluted 1:10 in distilled water to a final volume of 4.5 ml. A sample was removed to assess its killing activity against the sensitive strain *C.glabrata* (S-388) using a microtitre assay (A1). The solution was mixed with 0.2g (equivalent to a 1 ml bed volume) of pre-swollen Sephadex G-25 and incubated for one hour at 18^{o}C. The slurry was centrifuged for 10 minutes at 4,500 rpm and the supernatant removed for assay (A2). The Sephadex was washed with 4.5 ml of distilled water for 30 minutes with continuous stirring. The supernatant was removed (A3) and the Sephadex washed for a second time with distilled water (A4), followed by a salt wash with 0.1 M NaCl. The supernatant (A5) was assayed for killing activity and the effect of 0.1 M NaCl (A6) on the assay system also determined.

2.11 Mode of Action of *W.mrakii* (K-500) Killer Toxin Against Sensitive Yeast Strains

2.11.1 Effect of Growth Phase on Sensitivity to Killer Toxin

Stationary phase cells (5 ml) of an overnight culture of *C.glabrata* were inoculated into fresh SLM (50 ml) and incubated, with shaking, at 30^{o}C. At one hourly intervals, over the normal growth cycle, cells were removed and their sensitivity to *W.mrakii* killer toxin assessed using a microtitre assay. Increasing volumes (10-100 µl) of toxin preparation were made up to 100 µl with sterile distilled water and added to an equal volume of SLM seeded with 1x10^{5} cells/ml of the sensitive strain. The microtitre plate was incubated at 30^{o}C for 18-20 hours and the absorbance of each measured at 450 nm using an automatic plate reader.

The percentage reduction in growth of each phase of cells produced by increasing volumes of toxin was calculated (section 2.6.3) and used to plot 'sensitivity profiles'.

2.11.2 Effect of Killer Toxin on Growth Kinetics in the Sensitive Yeast

Three strains of *C.albicans*, 2005E, 2402E and C316, were incubated in the wells of a microtitre plate with increasing amounts of crude *W.mrakii* killer toxin, as above. The

plate was placed in an automatic spectrophotometer (Titertek Multi-Scanner) which monitored the growth of the cells in each well by measurement of the absorbance at 450 nm at 15 minute intervals over a 24 hour period. The plate was mixed at the same interval prior to growth measurements.

The information was collated *in situ* to produce a growth curve for each individual well. The growth rates of each sensitive strain in the presence of increasing amounts of the killer factor was determined.

2.11.3 Effect on Sensitive Cell Viability

The sensitive strain *C.glabrata* (S-388) was grown overnight in SLM at 30^{o}C. A 5 ml sample of cells was transfered to fresh SLM and grown in a Gallenkamp orbital incubator (140 rpm at 30^{o}C) until mid-log phase was reached. Cells were harvested by centrifugation (4,000 rpm for 5 minutes) and resuspended at a cell density of $3x10^{7}$ cells/ml in a concentrated toxin solution (crude freeze-dried material reconstituted in YNBGS). Over a period of 64 hours at 18^{o}C, samples were removed and the number of viable cells was determined by colony forming activity on SDA plates after a 10^{5} dilution of the cell suspension. The percentage of viable cells remaining in suspension was measured by comparison with colony forming units (CFU) from a toxin-free control.

Toxin-binding to sensitive cells was assessed by measurement of the killer activity remaining in the supernatant after removal of cells by centrifugation (12,000 rpm for 2 minutes). The degree of toxin binding was expressed as a percentage of the initial killer activity in the supernatant.

Residual activity of the concentrated toxin solution was monitored at room temperature over the period of incubation to confirm that there was no loss of activity under these conditions.

CHAPTER THREE

Broad Spectrum Activity of the Killer Yeast *Williopsis mrakii*

3.1 Introduction

Killer yeasts secrete toxins which are lethal or inhibitory to other sensitive yeast strains but to which they themselves are immune. The killer phenomenon, which was previously thought to be a specific yeast-yeast interaction, has been observed among unrelated eukaryotic microorganisms and bacteria (Polonelli and Morace, 1986; Morace *et al.*, 1989). However, this broad spectrum of action affords considerable interest in the use of killer toxins not only as anti-yeast preparations but also as a diagnostic system for yeast isolates.

One of the main objectives of this research was to identify killer yeast strains which suppressed or killed clinically important *Candida* species. Initially, work concentrated on the screening of yeasts to identify potential killer strains, and to further investigate their spectrum of activity. The yeast, *Williopsis mrakii* (K-500) displayed a broad spectrum of action against yeast of several genera and was consequently chosen for further study.

3.2 Determination of the Killer Character of Yeasts Held in the Culture Collection

A search was conducted for killer strains among certain yeast species held in the Institute's culture collection: the spore-forming ascomycetes (*Pichia, Schizosaccharomyces, Saccharomyces, Kluyveromyces, Williopsis* and *Debaryomyces*) and the asporogenous yeasts (*Candida* and *Phaffia*). The activity of the presumptive killers was assessed using a standard methylene blue agar diffusion bioassay (see section 2.6.1). The agar was seeded with the sensitive strain and each killer strain tested was streaked on the surface. After incubation, a positive result was indicated by a zone of clearing around the killer, bounded by stained blue (*i.e.* dead) colonies, or colonies of reduced size. The potential killers were tested against several strains of *S.cerevisiae* and *C.glabrata*, which had known sensitivities to killer yeasts and their toxins, and against clinical isolates of *Candida* from vaginal swabs. Plates were scored using the visual system outlined in section 2.6.1 and the results recorded in Tables 3.1a and 3.1b.

In all 21 killers were found among the 24 strains tested and several of these possessed high activity and a broad spectrum of action. The strongest killing was observed in

Table 3.1a - Screening of yeasts held in the culture collection for killer character using the agar diffusion bioassay.

KILLER STRAINS	SENSITIVE STRAINS					
	C.glabrata	Candida isolates			S.cerevisiae	
	S-388	21101F	214392	21408J	1006	381
P.anomala (K-432)	+++ 2	++ 1	++ 1	- 0	++ 2	++++ 0
P.anomala (K-434)	++ 1	++ 1	+ 1	(+) 1	+ 2	++ 1
P.anomala (K-435)	++ 1	+ 1	+ 1	- 1	+ 1	+++ 1
P.anomala (K-750)	++ 2	(+) 1	- 0	- 0	++ 1	(+) 0
P.membranae-faciens (K-333)	++ 2	(+) 1	- 1	- 1	+++ 2	(+) 0
P.subpellicosa (K-436)	- 1	n.d	n.d	n.d	n.d	n.d
W.californica (K-496)	+++ 2	n.d	n.d	n.d	n.d	n.d
W.mrakii (K-LKB)	++++ 2	(+) 0	+ 1	- 0	+++ 2	+++ 2
W.mrakii (K-500)	+++++ 2	+++ 2	+++ 2	++ 1	+++++ 2	++++ 2
W.saturnus (K-22)	- 0	- 1	- 0	- 0	(+) 1	- 0
W.saturnus (K-23)	- 0	- 0	+ 1	- 0	++ 1	+ 0
W.saturnus (K-979)	n.d	(+) 1	- 1	- 1	+++ 2	n.d
D.vanrijii (K-577)	(+) 1	- 0	(+) 1	- 1	- 0	- 1

For key to symbols see Table 3.1b.

Table 3.1b - Screening of the yeasts held in the culture collection for killer character using the agar diffusion bioassay.

KILLER STRAINS	SENSITIVE STRAINS					
	C.glabrata	Candida isolates			S.cerevisiae	
	S-388	21101F	214392	21408J	1006	381
K.drosophilarum (K-575)	n.d	(+) 1	(+) 1	+ 1	+++ 2	n.d
K.fragilis (K-1425)	- 0	- 0	- 1	- 1	- 1	- 1
K.marxianus (K-587)	(+) 1	- 0	(+) 1	- 1	(+) 0	++ 1
S.cerevisiae (K-28)	++ 1	(+) 1	(+) 1	+ 1	- 1	+ 1
S.cerevisiae (K-679)	- 0	- 0	+ 1	- 0	- 0	- 0
S.cerevisiae (K-761)	- 1	- 1	- 1	- 1	- 0	+ 0
S.cerevisiae (K-1001)	++ 1	+ 1	- 0	- 1	- 0	++ 2
S.cerevisiae (K-1109)	- 0	- 0	- 0	(+) 1	- 1	++ 2
S.pombe (K-1354)	- 0	- 1	- 1	- 1	- 1	- 1
P.rhodozyma (K-PR1)	(+) 1	+ 1	(+) 1	(+) 1	(+) 1	(+) 1
C.glabrata (S-388)	n.d	- 0	- 0	- 0	- 1	n.d

KEY : - indicates no killer activity and + to +++++ is indicative of trace to good killer activity. (+) signifies an undefined ("fuzzy") zone of clearing, **0** an absence of blue-stained colonies, **1** stained colonies beneath the streak only and **2** a distinct border of stained colonies. In some cases killer character was not determined (n.d).

species of the genera *Pichia* and *Williopsis* and it is apparent from the results that *W.mrakii* (K-500) was the predominant strain, showing moderate to extensive killer activity against all the sensitive strains tested. Plate 3.1 shows the typically large zones of inhibition produced and, in all but one case, there was an accumulation of methylene blue around this lethal zone. A second strain of *W.mrakii* (K-LKB) displayed a narrower range of activity, being active against *C.glabrata* (S-388) and the strains of *S.cerevisiae*, yet it showed little or no activity against the clinical isolates of *Candida*. In general only weak killer activity was displayed by strains of *W.saturnus*. They too, were largely inactive against the strains of *Candida*, but showed a degree of activity against *S.cerevisiae* (S-1006). With the exception of *P.subpellicosa* (K-436), all isolates of *Pichia* showed good killer activity. The 4 strains of *P.anomala* assayed showed quite different patterns of activity and the strongest killers were K-432 and K-434. However, in common with *P.membranaefaciens* (K-333), all were more active against strains of *S.cerevisiae*. Similarly, *Kluyveromyces* species were also largely inactive against the *Candida* strains, but *K.drosophilarum* (K-575) and *K.marxianus* (K-587) showed weak to moderate activity against S-1006 and S-381, respectively. Strains of *S.cerevisiae* tended to show intrageneric, rather than interspecific killing activity. All of these strains were more active against S-381 than S-1006, however, both K-28 and K-1001 showed weak activity against *C.glabrata* (S-388). *D.vanrijii* (K-577) showed only trace activity against S-388 and the clinical isolate 214392 and *P.rhodozyma* (K-PR1) displayed signs of activity against all sensitive strains tested. No toxic effects were displayed by *S.pombe* (K-1354), *K.fragilis* (K-1425) and *C.glabrata* (S-388).

Of the seeded strains used, *C.glabrata* (S-388) and *S.cerevisiae* (S-381) were most sensitive to the action of the killer yeasts, displaying a response to 13 and 14 of the killers respectively. None of the sensitive strains tested were resistant to all of the killer strains, however, the clinical isolate 21408J, showed the least susceptibility to their toxic action.

Plate 3.1 - Growth inhibition of the sensitive strain *C.glabrata* (S-388) by the killer yeast *W.mrakii* (K-500).

1 = *Williopsis* killer yeast, **2** = *Candida* sensitive, **3** = non-killer yeast.

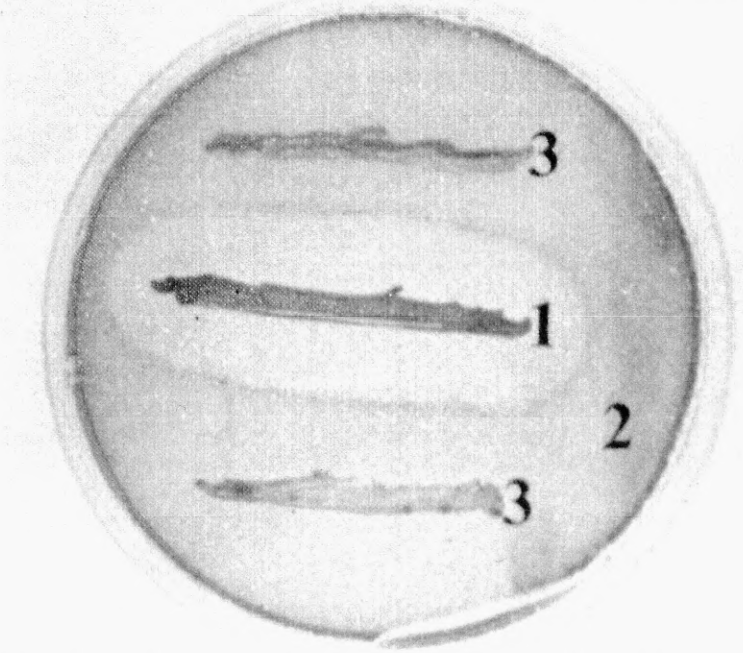
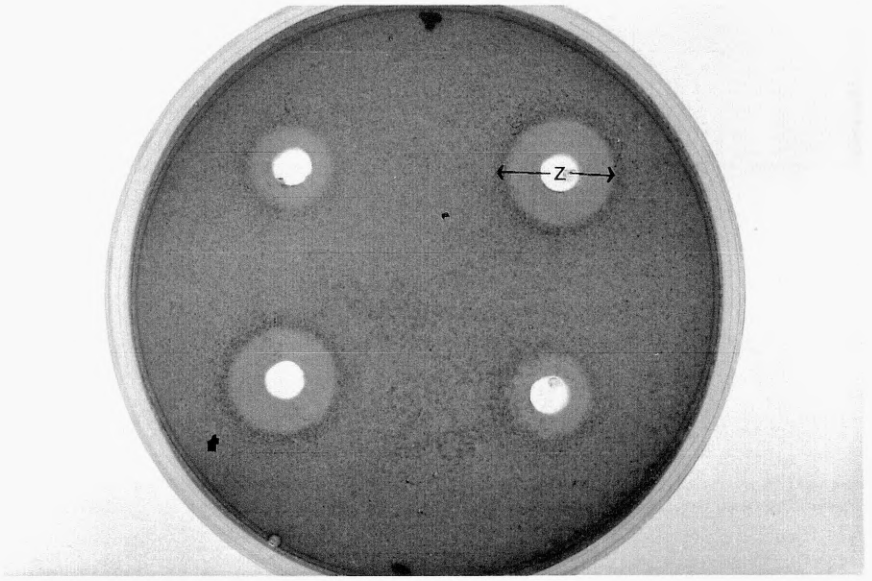


Plate 3.2 - Agar diffusion well bioassay to show the effects of 50 µl of cell-free supernatant from 48 hour batch cultures of *W.mrakii*. Mean zones of inhibition (**z**) were measured to the nearest 1.0 mm from 5 replicate wells.



3.3 Classification of Killer Yeasts Based on Cross-Reaction Studies

Possible killer-sensitive relationships of a selection of killer yeasts (see Table 3.2) were examined using a streak-plate bioassay. Each killer yeast was tested for its ability to kill and for its sensitivity to the killing activity of each other killer strain. The assay medium (section 2.6.1) was seeded with an appropriate volume of an overnight culture of each killer and samples of the killer yeasts were taken from SDA plates and streaked on the surface. After incubation they were scored for the presence (+) or absence (-) of killing activity (Table 3.3).

From the results it can be seen that there were nine distinct patterns of killer activity against other killer yeasts, designated K_a-K_i (Young and Yagui, 1978). All of the *S.cerevisiae* strains were found in the first two killer types, with none of the strains showing any other type of activity. Those yeast in type K_a were active only against another strain of *S.cerevisiae*, K-28 whereas type K_b were inactive against all strains tested. This killer phenotype was also shown by *K.marxianus* (K-587). Type K_c was shown by a strain of *W.mrakii* (K-LKB), which was active against six of the other killers, five strains of *S.cerevisiae* and *K.marxianus* (K-587). Two strains of *P.anomala* (K-432 and K-435) showed the same killer phenotype K_f, and were active against a single strain of *K.drosophilarum* (K-575). *W.mrakii* (K-500), type K_h, showed the most extensive activity against seven of the 12 killers tested. With the exception of killing *P.anomala* (K-435), the spectrum of activity of this strain was identical to *W.mrakii* (K-LKB). Types K_d, K_e, K_g and K_i were shown by the single strains *P.anomala* (K-434), *P.anomala* (K-750), *K.drosophilarum* (K-575) and *K.lactis* (K-1368) respectively, each displaying a different spectrum of activity.

Results in Table 3.3 also demonstrate the resistance to killer action of the different killer yeasts, and ten patterns of resistance were observed (R_a-R_j). K-500 and K-1368 (R_j) were resistant to the action of all of the killers, and the remaining strains showed resistance to at least nine of the others. Yeasts which demonstrated the same killer phenotype (K_a, K_b and K_f) all exhibited different resistance patterns (1006, K_aR_a; 28, K_bR_b; 1001, K_bR_c; 587, K_bR_h; 435, K_fR_g). The resistant phenotypes of K-232 and K-761 were not determined because of their failure to grow as seeded strains and K-432

Table 3.2 - Killer yeasts used in cross-reaction studies.

Genus	Species	Code Number	Source
Saccharomyces	cerevisiae	K-28	Dr. Pfeiffer
		K-232	N.C.Y.C
		K-761	N.C.Y.C
		K-1001	N.C.Y.C
		K-1006	N.C.Y.C
Pichia	anomala	K-432	N.C.Y.C
		K-434	N.C.Y.C
		K-435	N.C.Y.C
		K-750	N.C.Y.C
Williopsis	mrakii	K-500	N.C.Y.C
		K-LKB	Dr. Kodama
Kluyveromyces	drosophilarum	K-575	N.C.Y.C
	lactis	K-1368	N.C.Y.C
	marxianus	K-587	N.C.Y.C

N.C.Y.C - National Collection of Yeast Cultures, Redhill, Surrey, England.

Cultures were kindly donated by Dr. Pfeiffer, Institut fur Mikrobiologie und Weinforschung, Germany and Dr. Kodama, Laboratory of Kodama Brewing Co. Ltd., Japan.

Table 3.3 - Killer / sensitive relationships of selected killer yeast strains.

Tested Yeast Strains	Seeded Yeast Strains														Killer Type
	1006	28	1001	LKB	434	432	750	435	587	575	500	1368	232	761	
	R_a	R_b	R_c	R_d	R_e		R_f	R_g	R_h	R_i	R_j		n.d		
1006	-	+	-	-	-	-	-	-	-	-	-	-	n.d	n.d	K_a
761	-	+	-	-	-	-	-	-	-	-	-	-	n.d	n.d	
28	-	-	-	-	-	-	-	-	-	-	-	-	n.d	n.d	K_b
1001	-	-	-	-	-	-	-	-	-	-	-	-	n.d	n.d	
587	-	-	-	-	-	-	-	-	-	-	-	-	n.d	n.d	
232	-	-	-	-	-	-	-	-	-	-	-	-	n.d	n.d	
LKB	+	+	-	-	+	+	+	-	+	-	-	-	n.d	n.d	K_c
434	-	-	+	+	-	-	-	-	-	+	-	-	n.d	n.d	K_d
750	-	-	+	-	-	-	-	-	-	+	-	-	n.d	n.d	K_e
432	-	-	-	-	-	-	-	-	-	+	-	-	n.d	n.d	K_f
435	-	-	-	-	-	-	-	-	-	+	-	-	n.d	n.d	
575	+	-	-	-	-	-	-	-	-	-	-	-	n.d	n.d	K_g
500	+	+	-	-	+	+	+	+	-	+	-	-	n.d	n.d	K_h
1368	-	-	-	-	-	-	+	-	-	-	-	-	n.d	n.d	K_i

+ Killing activity; - No killing activity; K_a-K_i Killer phenotype;

R_a-R_j Resistance phenotype; **n.d** not determined

had the resistance pattern (R_e) in common with K-434. The remaining three yeast strains displayed unique killer and resistant phenotypes (LKB, K_cR_d; 750, K_eR_f and 575, K_gR_i). All of the killer yeasts screened were immune to the action of their own toxin and *K.drosophilarum* (K-575) was most sensitive to the action of the other killer yeast.

3.4 Anti-*Candida* Activity of *W.mrakii* Killer Yeasts

Clinical isolates of *Candida* were used to further assess the potential of killer yeasts and their toxins as antifungal agents against pathogenic yeast strains.

An initial screen of the activity of *W.mrakii* (K-500) killer yeast against a small number of isolates (obtained from Ninewells Hospital, Dundee) was conducted using the agar diffusion and microtitre bioassays (see sections 2.6.1 and 2.6.3). The isolates included those from vaginal swabs, lung and tracheal aspirates and blood and urine samples. The killer activity expressed against each isolate (see Tables 3.4a and 3.4b) was compared to the effect of the killer strain and its toxin on the standard sensitive, *C.glabrata* (S-388).

The results further emphasised the differential killing activity of the yeast *W.mrakii* and highlight the potential action of killer toxin preparations against medically important yeast strains. Results from the two bioassays produced very similar responses between the killer yeast, its toxin and the sensitive isolate being tested. Results in Table 3.4a showed that the killer yeast was most active against the lung and tracheal aspirates, causing pronounced inhibition of their growth in the agar diffusion bioassay. Isolate S-00659H from a nasal swab, was also relatively sensitive to the action of K-500. Two of the three vaginal isolates were essentially resistant to the killer yeast, however, isolate S-214392 displayed susceptibility to its action. An isolate from a blood sample, identified as *C.pseudotropicalis*, was also resistant to its action.

The microtitre bioassays (Table 3.4b) similarly showed that vaginal *Candida* were resistant to the action of the killer toxin, showing less than 10% reduction in their growth (see Figure 3.1). Isolates taken from blood and nasal swabs showed little or no sensitivity (Figure 3.2), however, lung and tracheal isolates were sensitive to toxin action resulting in 40-65% reduction of their growth (Figure 3.3).

Table 3.4a - Results of an agar diffusion bioassay showing the differential sensitivity of clinical isolates to the killer yeast *W.mrakii* (K-500).

Seeded Strain	Clinical Source	Killer Sensitivity
S-01563X	Tracheal aspirate	+++ 2
S-21408J	Vaginal swab	- 0
S-00659H	Nasal swab	++ 2
S-214392	Vaginal swab	+++ 2
S-07278S	C.pseudotropicalis blood sample	- 1
S-018179	Lung aspirate	+++ 2
S-091834	Tracheal aspirate	+++ 2
S-21101F	Vaginal swab	+ 2
S-388	Standard sensitive	+++++ 2

KEY: - indicates no killer activity and + to +++++ is indicative of trace to good killer activity. **0** signifies an absence of blue-stained colonies, **1** stained blue colonies beneath the streak only and **2** a distinct border of stained colonies.

Table 3.4b - Results of a series of microtitre bioassays showing the percentage reduction in growth produced by *W.mrakii* (K-500) killer toxin against clinical isolates of *Candida.*

Seeded Strain	% Reduction in Growth of the Seeded Strains Produced by K-500 Killer Toxin With Respect to Total Amount of Protein (µg) per Well of Assay					
	0.120	0.240	0.336	0.384	0.432	0.480
C.glabrata (S-388)	0.1	2.9	95.0	94.1	94.1	90.5
Tracheal aspirate (S-01563X)	13.7	4.6	17.6	38.5	52.9	69.6
Vaginal swab (S-21408J)	5.2	3.8	5.9	10.1	7.3	9.8
Nasal swab (S-00659H)	10.3	10.9	14.0	18.8	25.1	25.4
Vaginal swab (S-214392)	5.3	9.2	8.0	8.7	11.9	14.6
Blood sample (S-07278S)	2.8	1.5	0	0.4	0.7	5.0
Lung aspirate (S-018179)	5.2	6.2	18.2	38.1	45.2	61.0
Tracheal aspirate (S-091834)	1.7	2.0	2.4	31.0	40.0	45.0
Vaginal swab (S-21101F)	5.5	7.2	5.8	7.2	8.9	11.9

Figure 3.1 - Effect of *W.mrakii* (K-500) killer toxin on vaginal isolates of *Candida*. The percentage reduction in growth of each isolate was measured using a microtitre assay and the response compared to the standard sensitive strain, *C.glabrata* (S-388).

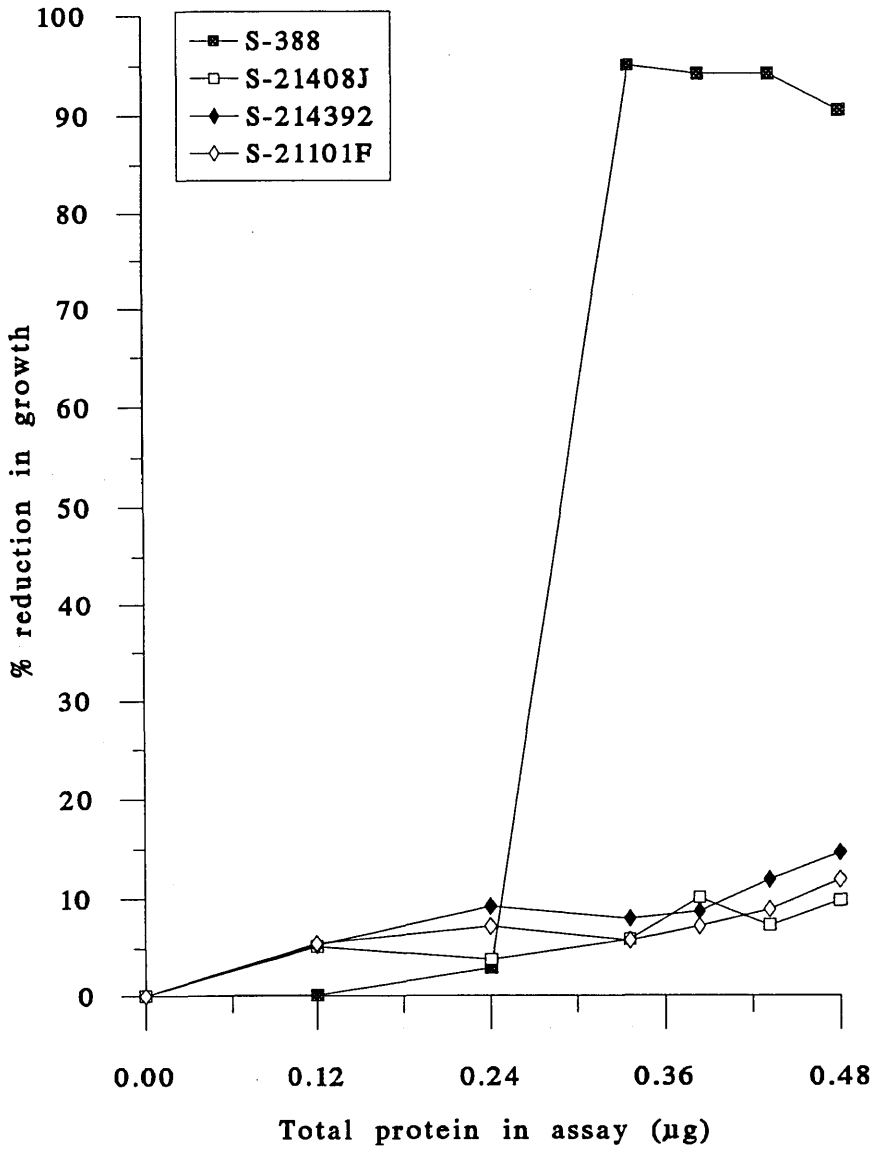


Figure 3.2 - Effect of *W.mrakii* (K-500) killer toxin on lung and tracheal isolates of *Candida*. The percentage reduction in growth of each isolate was measured using a microtitre assay and the response compared to the standard sensitive strain, *C.glabrata* (S-388).

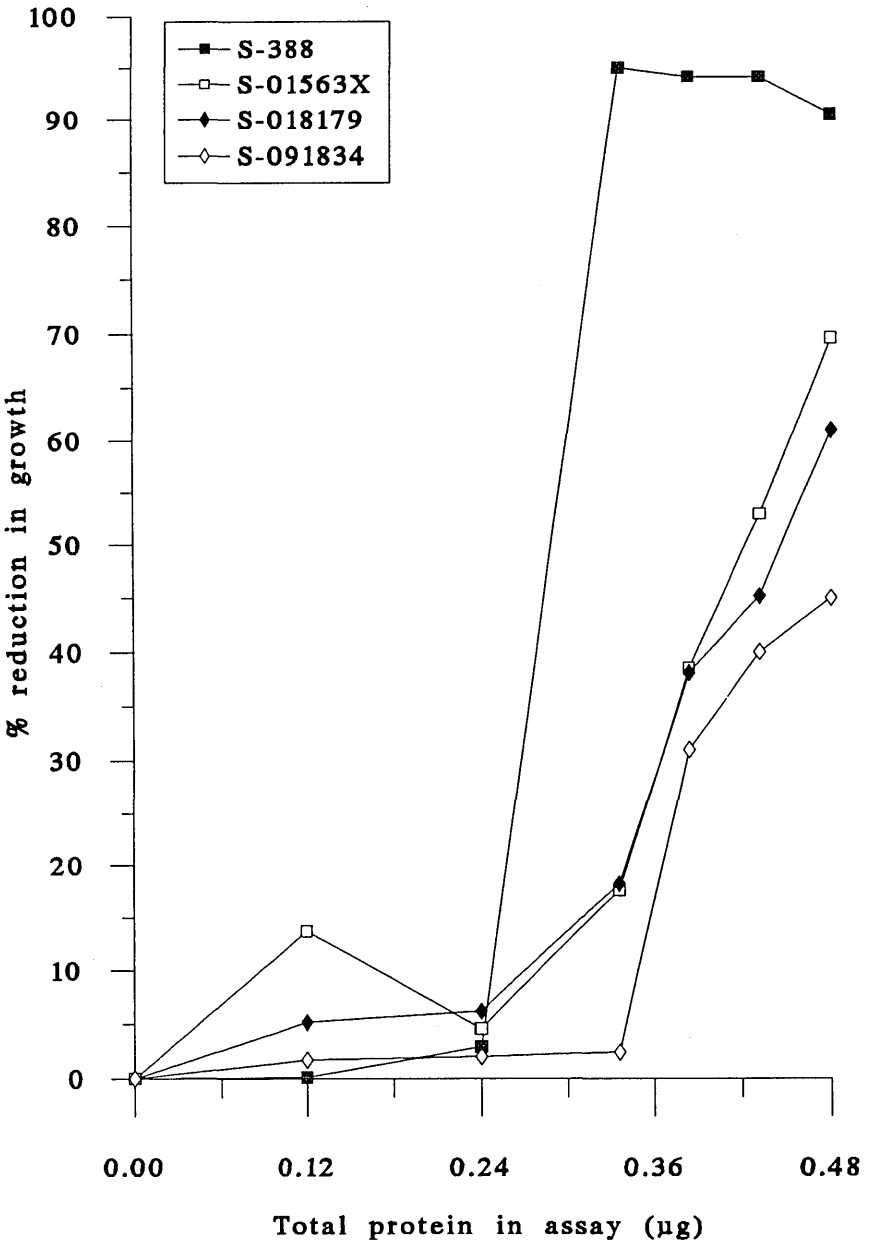
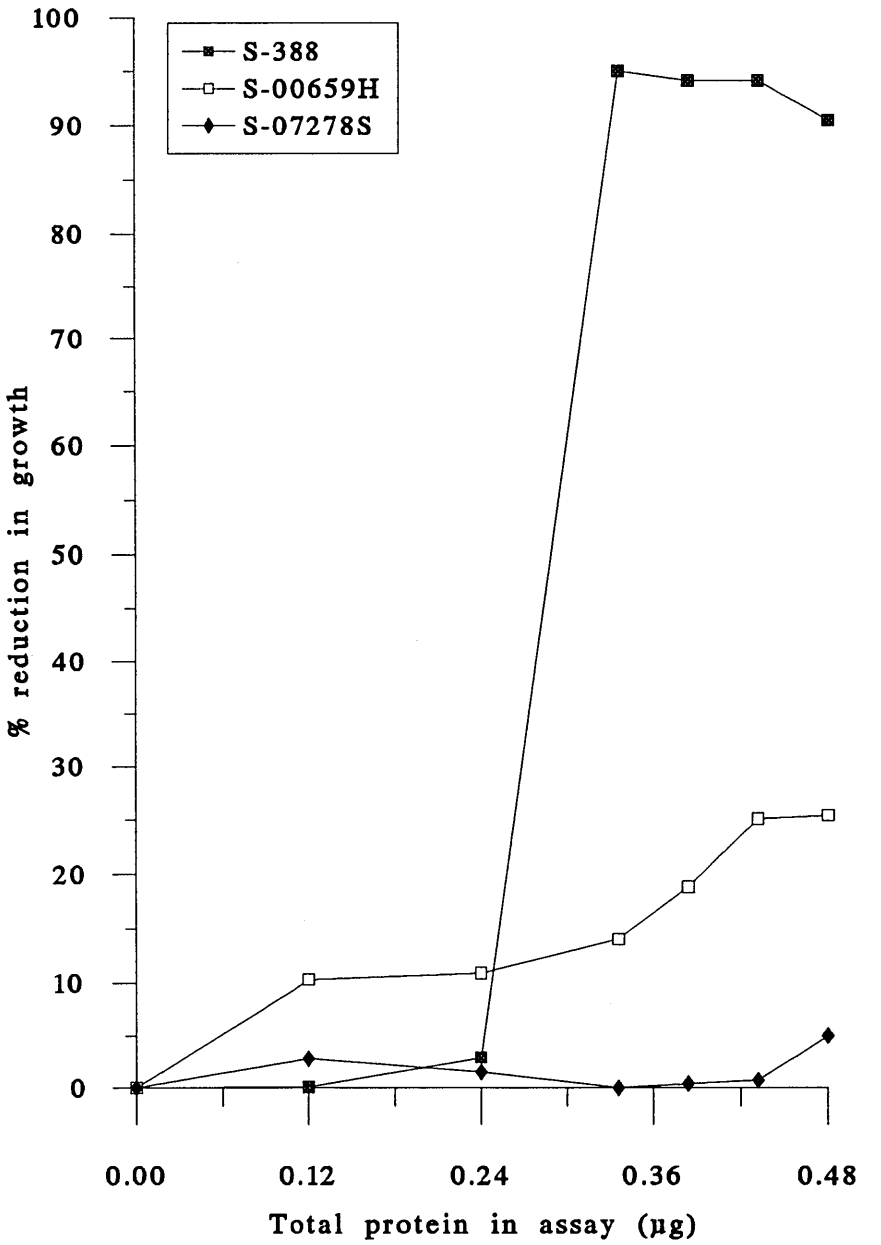


Figure 3.3 - Effect of *W.mrakii* (K-500) killer toxin on nasal (00659H) and blood (07278S) isolates of *Candida*. The percentage reduction in growth of each isolate was measured using a microtitre assay and the response compared to the standard sensitive strain, *C.glabrata* (S-388).



These results suggested a possible relationship between the sensitivity of the isolates to *W.mrakii* (K-500) killer yeast, and its toxin, and their physiological niche. A more extensive screen was performed to investigate this hypothesis further. The effect of crude toxin preparations (cell-free supernatants from a 4 day static culture) of two *W.mrakii* killer strains, K-500 and K-LKB, on 51 clinical isolates of *Candida* was investigated using the agar diffusion well bioassay (section 2.6.2) and microtitre bioassay (section 2.6.3) and the results compared to a streak-plate agar diffusion bioassay (section 2.6.1). The isolates from the initial study were re-assayed in conjunction with further samples obtained from Ninewells Hospital.

Results obtained from the series of bioassays (Tables 3.5a, 3.5b, 3.6a and 3.6b) confirmed that the majority of vaginal isolates tested were resistant to the action of both K-500 and K-LKB killer yeast strains. In microtitre assays (Table 3.5a) eight of the isolates tested against K-500 showed less than 20% reduction in their growth, and a further four isolates displayed less than 40% reduction. 13 isolates tested against K-LKB showed little or no susceptibility to the toxin (Table 3.6a). Results from a well bioassay (Table 3.5b) displayed similar responses to the action of the killer toxins, however, the streak-plate bioassay suggested that as many as 14 isolates were resistant to K-500 killer yeast. Only a very weak killer activity by K-LKB was demonstrated against the same number of isolates (Table 3.6b).

Microtitre assays (Table 3.5a) showed that all aspirate isolates tested displayed moderate sensitivity to the K-500 killer toxin, with a range of 37-63% reduction in their growth. This compared well with the initial screen where a range of 40-67% was observed (Table 3.4b). The agar bioassays confirmed these findings, and further emphasised that no isolate was completely resistant to toxin action. A different spectrum of activity was displayed by K-LKB and the isolates were, on the whole, less sensitive to this killer yeast and its toxin.

The response of sputum isolates to the action of K-500 killer toxin in microtitre and well bioassays was less pronounced, a broad range of sensitivity to its action was observed. The majority of the isolates showed a resistance to the killer yeast in the streak-plate bioassays. However, in each assay four isolates were consistently seen to be highly susceptible to the toxin. In particular isolate 26602K, which was completely inhibited by

Table 3.5a - Results of a series of microtitre assays showing the effects of *W.mrakii* (K-500) killer toxin on clinical *Candida*. Figures are relative to the total protein in the assay (0.52µg).

Clinical Isolates	No. of Isolates Tested	No. Showing % Reduction in Growth			
		0-20%	21-50%	51-70%	71-100%
Aspirates	5	0	3	2	0
Vaginal[a]	17	8	6	1	2
Sputum	15	4	3	4	4
Oral[b]	7	2	3	1	1
Blood[c]	2	1	0	0	1
Oesophageal	1	0	1	0	0
Ear	1	0	1	0	0
Urethral	1	0	0	0	1
Abdominal	1	0	0	0	1
Urine	1	0	0	0	1

a - this includes both vaginal swabs and endocervical smears

b - this includes both mouth and throat swabs

c - isolates previously identified as *C.pseudotropicalis* and *C.parapsilosis*

Table 3.5b - Results of a series of streak-plate and well bioassays showing the numbers of clinical isolates of *Candida* which were sensitive to the action of the yeast *W.mrakii* (K-500) and its killer toxin.

Clinical Isolates	No. of Isolates Tested	Sensitivity Patterns of Streak-Plate Bioassays			Sensitivity Patterns of Well Bioassay (zone of inhibition mm)		
		resistant	weak	sensitive	0-4	5-8	9-12
Aspirates	5	0	5	0	0	3	2
Vaginal	17	14	3	0	9	7	1
Sputum	15	11	1	3	6	5	4[a]
Oral	7	6	0	1	3	3	1
Blood	2	2	0	0	2	0	0
Oesophageal	1	1	0	0	0	1	0
Ear	1	1	0	0	1	0	0
Urethral	1	1	0	0	0	0	1
Abdominal	1	1	0	0	0	0	1
Urine	1	1	0	0	0	1	0

a - sputum isolate 26602K showed a zone of inhibition of 22.0mm

Table 3.6a - Results of a series of microtitre assays showing the effects of *W.mrakii* (K-LKB) killer toxin on clinical *Candida*. Figures are relative to the total protein in the assay (0.56µg).

Clinical Isolates	No. of Isolates Tested	No. Showing % Reduction in Growth			
		0-20%	21-50%	51-70%	71-100%
Aspirates	5	2	3	0	0
Vaginal[a]	17	13	2	0	2
Sputum	15	9	2	0	4
Oral[b]	7	4	1	0	2
Blood[c]	2	1	0	0	1
Oesophageal	1	0	1	0	0
Ear	1	0	1	0	0
Urethral	1	0	0	0	1
Abdominal	1	0	0	0	1
Urine	1	0	0	0	1

a - this includes both vaginal swabs and endocervical smears

b - this includes both mouth and throat swabs

c - isolates previously identified as *C.pseudotropicalis* and *C.parapsilosis*

Table 3.6b - Results of a series of streak-plate and well bioassays showing the numbers of clinical isolates of *Candida* which were sensitive to the action of the yeast *W.mrakii* (K-LKB) and its killer toxin.

Clinical Isolates	No. of Isolates Tested	Sensitivity Patterns of Streak-Plate Bioassays			Sensitivity Patterns of Well Bioassay (zone of inhibition mm)		
		resistant	weak	sensitive	0-4	5-8	9-12
Aspirates	5	1	4	0	3	1	1
Vaginal	17	1	13	3	12	5	0
Sputum	15	5	6	4	9	3	3[a]
Oral	7	3	3	1	6	1	0
Blood	2	2	0	0	2	0	0
Oesophageal	1	1	0	0	1	0	0
Ear	1	1	0	0	1	0	0
Urethral	1	0	1	0	0	1	0
Abdominal	1	0	0	1	0	1	0
Urine	1	0	1	0	0	1	0

a - sputum isolate 26602K showed a zone of inhibition of 21.5mm

the toxin in microtitre assays, and produced a zone of killing of 22 mm in a well bioassay. In general, K-LKB showed little activity against the isolates which were sensitive to K-500 killer toxin in microtitre and well assays, yet a broad spectrum of action in agar diffusion bioassays. The elevated response of 26602K to K-LKB toxin was in common with that seen with the K-500 killer yeast strain.

Oral isolates (from throat and mouth swabs) displayed a weak sensitivity to the action of K-500 killer toxin with only two of the seven isolates tested showing greater than 50% reduction in growth. The results from agar bioassays confirmed the limited killer activity of K-500 against these isolates. Similar patterns of sensitivity to K-LKB were observed with the killer toxin producing greater than 70% reduction in growth in only two of the isolates. A greater response was seen to the killer yeast in the streak-plate assay than killer toxin in a well bioassay.

Two isolates from blood, previously identified as *C.pseudotropicalis* and *C.parapsilosis*, were tested for their sensitivity to both K-500 and K-LKB killer yeasts. In agar bioassays neither isolate was sensitive to their action, however, the killer toxins produced quite different responses in microtitre assays. *C.pseudotropicalis* showed little sensitivity to K-500 killer toxin (10% reduction in growth) but was very susceptible to K-LKB toxin, which completely inhibited its growth. *C.parapsilosis*, however, was moderately sensitive to K-500 (50% reduction in growth) but completely resistant to K-LKB.

Only single isolates from the oesophageal, ear and urethral swabs, abdominal drain sites and urine were available for assay. Identical patterns of sensitivity to the two killer toxins was observed in microtitre assays. In streak-plate bioassays the isolates from the abdomen, urethra and urine were more sensitive to the killer yeast K-LKB, whereas in the well bioassay isolates from the abdomen, oesophagus and urethra were more susceptible to the action of K-500 killer toxin.

3.5 Identification of Clinical Isolates of *Candida*

Isolates received from Ninewells Hospital were identified only as strains belonging to the genus *Candida*, with the exception of the two isolates taken from blood samples which had been formerly identified as *C.pseudotropicalis* and *C.parapsilosis*. Attempts were subsequently made to characterise the remaining isolates and further investigate the relationship between toxin action and isolate sensitivity.

Microring YT is an *in vitro* diagnostic testing system and was used to further identify the clinical isolates (see section 2.2). Conventional methods for the identification of yeasts rely on fermentation and assimilation tests. Microring YT identifies unknown organisms by comparison of their susceptibility patterns to the various agents on the ring with a table of known susceptibility profiles. Microrings carry six tips which are impregnated with a range of antimicrobial agents and dyes. Each tip performs as an individual disc test because of an isolating hydrophobic barrier. SDA plates were inoculated with each isolate and the rings placed securely on the agar surface. After incubation at 37^{0}C, susceptibilities were read at 24 and 48 hours, and the subsequent profiles were compared to those of known yeast isolates.

Results in Table 3.7 showed that 73% of the isolates used in previous studies were positively identified using the commercial YT Microrings. Of the 37 isolates identified, 32 were characterised as strains of *C.albicans*, 2 as *C.glabrata*, 2 as *C.parapsilosis* and 1 as *C.pseudotropicalis*. The 14 remaining isolates were not conclusively identified using this system, in several cases the Microrings failed to distinguish between a number of possible species.

Table 3.7 - Table showing the identification of *Candida* species isolated from various sites of the body. Isolates were identified using the Microring YT *in vitro* diagnostic testing system which identifies unknown organisms by their susceptibilities to various antimicrobial agents and dyes.

Site of Isolation	No. of Isolates	C.albicans	C.glabrata	C.pseudotropicalis	C.parapsilosis	Unknown
Aspirates	5	5	0	0	0	0
Vaginal	17	11	1	0	0	5
Sputum	15	10	1	0	1	3
Oral	7	4	0	0	0	3
Blood	2	0	0	1	1	0
Oesophageal	1	1	0	0	0	0
Ear	1	0	0	0	0	1
Urethral	1	1	0	0	0	0
Abdominal	1	0	0	0	0	1
Urine	1	0	0	0	0	1

3.6 Discussion

The killer phenomenon in yeast was first described for a number of laboratory strains of *S.cerevisiae* (Bevan and Makower, 1963). Killer strains released a substance which produced a lethal response in sensitive strains (Woods and Bevan, 1968). Several studies have since been conducted to determine the occurrence of killer character in yeasts of various genera which were obtained from laboratory culture collections (Philliskirk and Young, 1975), natural habitats (Stumm *et al.*, 1977), fermentation processes (Rosini, 1983) and clinical specimens (Kandel and Stern, 1979). Killer strains have consequently been reported in a number of genera (see section 1.1).

It was evident from the results in section 3.2, that vast differences exist between killer yeasts both in terms of the extent of killing produced and the range of organisms which are sensitive to the action of the toxin. It was also clear that the killer trait was not specific to any one particular genera or species. Killers strains were found in six of the eight different genera tested from yeast held in the Institute's culture collection, only *Candida* and *Schizosaccharomyces* were not represented. The highest proportion of killers were found in the genera of *Pichia* and *Williopsis* (formerly taxonomically defined as *Hansenula*), 10 of the 12 strains assayed from the above genera displayed killer activity against the selected sensitive strains (Table 3.1a). This was in agreement with several earlier reports (Philliskirk and Young, 1975; Morace *et al.*, 1984; Kazantseva and Zimina, 1989). Although active against *Candida* isolates, intrageneric killing between strains of *S.cerevisiae* was also observed (Table 3.1b).

Apart from differences seen in the patterns of activity between species, considerable variability in killer activity was observed between strains, notably within *P.anomala*, *S.cerevisiae* and *W.mrakii*. The results in Tables 3.1a and 3.1b show that although similarities were seen between the strains in their action against specific sensitive strains, overall different patterns of activity were observed. Kazantseva and Zimina (1989) reported an 85% incidence of killers within strains of *P.anomala*, however, also observed very different spectra of activity.

Selection of putative sensitive strains to be used in an assay system holds great significance for the identification of killer activity in yeast strains. Consequently, results

from different studies will vary considerably depending on the selection of such strains. The sensitives *C.glabrata*, S-388 (Young and Yagui, 1978), *S.cerevisiae*, S-1006 (Philliskirk and Young, 1975) and *S.cerevisiae*, S-381 (Pfeiffer and Radler, 1982) were used in the screening of the culture collection because of their known sensitivity to a number of killer yeast. One of the main objectives of this research was to highlight a yeast strain with potential anti-*Candida* activity, therefore, three strains of *Candida* isolated from clinical specimens were also used as seeded indicator strains in the agar diffusion bioassays. An increase in the number of sensitive strains used, and representatives of different species and genera, would have undoubtedly led to different spectra of action being observed and perhaps would have highlighted additional killer yeasts.

The conditions of the bioassay used may have led to an underestimation in the numbers of killer strains. The assay employed in the initial screen was a modification of the streak-plate assay of Stumm *et al.* (1977). Media containing SLM and tryptone was used which was designed to prevent overgrowth of the sensitive strain, a problem associated with some strains of *Candida* (O'Leary, 1987). The growth medium also contained methylene blue dye, which is known to differentiate between live and dead colonies of *S.cerevisiae* (Woods and Bevan, 1968), and glycerol which has been reported to stabilise the killer factor (Lehmann *et al.*, 1987). The medium was buffered to pH 4.5, a standard used since the observations of Bevan and Makower (1963) revealed that killing activity was expressed only under acidic conditions. Attempts were made to optimise the assay conditions, however, it is possible that they may be sub-optimal for a number of the killers tested and the incubation temperature of 25^{o}C may be lower than the optimal growth temperature for either the sensitive or killer yeast strains. Some variation may exist in the amount of toxin that is produced by each killer strain when streaked on the surface of the agar plates. Although care was taken to streak single colonies of comparable size, each yeast may have showed different growth characteristics and in some cases produced a very small, if not undetectable amount of toxin. Therefore, poor growth of the streak may have led to some yeasts being given a lower score or in fact being scored as a non-killer.

Results in section 3.4 suggested that quite different patterns of activity were obtained if toxin preparations, rather than whole cell cultures, were used in assay systems. The

effect of the killer yeasts, K-LKB and K-500, against clinical *Candida* was investigated using a streak-plate assay, and the effect of their toxins using well and microtitre bioassays. The streak-plate bioassays provided a preliminary qualitative determination of the spectra of activity of the killer yeasts and the response of each isolate was scored visually (Table 3.5b and 3.6b). The assay was limited by the growth of the killer yeast on the surface of the plates and by the growth of the clinical isolates within the agar. Microtitre and well bioassays of the killer toxin preparations provided a quantitative and more critical assessment of killer activity, allowing a greater definition of the sensitivity of the isolates to the action of the killer toxins. Results from the two assays compared well, however, the microtitre assay provided a rapid (24 hours) assessment of killer toxin activity and was favoured to the more traditional agar diffusion bioassays (2-3 days) for future assessment of toxin action.

The diversity of action displayed by the killer yeasts suggests that the toxins produced by each may be biochemically distinct from one another. Several killer toxins of varying molecular sizes have, thus far, been characterised as being either proteins (*S.cerevisiae* K1 toxin, Palfree and Bussey, 1979) or glycoproteins (*P.kluyveri* killer toxin, Middelbeek *et al.*, 1979). It may also be possible that multiple toxins are capable of being produced by a single yeast strain in response to being challenged by different sensitives (O'Leary, 1987). In either case, the assay system may not compensate for differences in the composition of the toxin produced.

Expression of killer character by killer yeast strains results from the synthesis and excretion of killer toxin. If the nature of this killer factor is determined this will give an effective means of classification, since strains with identical patterns of action against sensitive strains will probably synthesize identical toxins. Cross-reaction studies allow interactions between killer yeasts to be analysed. Each killer yeast assayed was in fact immune to its own killer factor (Table 3.3) indicating that they possessed no site of action for their own toxin, or the site was unavailable for toxin binding. It is understandable that the killer strains are immune to the action of their own toxin so that when one killer yeast kills another, the killed strain has no immunity to the toxin to which it is susceptible, thus, the killer toxin must be biochemically distinct from that produced by the sensitive strain (Young, 1987). The results in Table 3.3 indicated that

nine of the 14 yeasts produced structurally distinct killer factors, and they were designated K_a-K_i.

The results of the cross-reaction studies were, only in part, in agreement with a similar study conducted by Young and Yagui (1978) which included several of the same killer strains (see Table 1.1). A similar grouping of *S.cerevisiae* strains was observed, however, in the earlier study these strains had a much broader pattern of activity, albeit against other strains of *S.cerevisiae*. In the present study K_a killers were found to be active only against *S.cerevisiae* (K-28), whereas, type K_b killers appeared to produce no, or undetectable amounts of killer factor. However, their activity against two further strains of *S.cerevisiae* (K-232 and K-761) was not determined. In both studies no intrageneric killing was seen between strains of *Pichia*, *Williopsis* and *Kluyveromyces*. However, a number of interactions were observed between the closely related strains of *Williopsis* and *Pichia*, which may indicate some similarity in the killer factor produced or in the receptors for toxin action on their cell surface.

The yeasts were also assigned the resistant phenotypes R_a-R_j. Several strains of the same killing group showed different resistance patterns and several strains belonging to different killing groups showed the same resistance patterns. The property of resistance is based on both immunity, which in the case of *S.cerevisiae* strains is plasmid-encoded (Somers and Bevan, 1969; Sweeney *et al.*, 1976) and for non-*Saccharomyces* strains encoded on nuclear genes (Young and Yagui, 1978), and also inherent differences in the composition of the cell wall (Young, 1987). The findings of the overall resistance observed in *W.mrakii* (K-500) to every other killer strain is in agreement with Young and Yagui (1978). On the other hand, *K.drosophilarum* (K-575) was the most sensitive to the action of the other killer factors, which contradicted their findings.

Nine killer phenotypes and 10 resistance phenotypes were recognised. Classification using both descriptions (*e.g.* *W.mrakii* K_hR_j) provides 12 unique categories for each killer tested (discounting K-232 and K-761 for which resistant phenotypes were not determined). Further examination of these and other killer yeasts, in all likelihood, would provide further categories and additional members to those described.

The activity of *W.mrakii* (K-500) killer yeast and its killer toxin was assessed against a small number of isolates from various sites of the body and the results suggested a

niche-specific response to the killer factor produced. The toxin was more active against lung and tracheal aspirates (45-70% reduction in growth) than nasal, blood or vaginal isolates (10-25% reduction in growth). This implied an inherent genetic variation and adaptation amongst opportunistic pathogenic flora. The little or no activity of *W.mrakii* killer toxin observed against the vaginal isolates may be due to their adaptation to the acidic conditions prevalent in the vagina. The pH in the vaginal lumen is controlled primarily by lactic acid produced from cellular glycogen or carbohydrates by the action of the normal bacterial microflora. The pH varies between 4.0 and 5.0 during the menstrual cycle and this acidity plays a clinically important role in preventing the proliferation of pathogenic strains (Okada, 1991). Under these conditions, strains of *Candida* may have undergone morphological changes and acquired a resistance, or loss of sensitivity, to the action of the killer toxin. Conditions encountered within the lung and tracheal cavities of the body, however, are less extreme and it is unlikely that aberrant morphological changes have resulted to enhance the survival of the *Candida* strains.

The expanded screen showed that both killer yeast strains were active, to varying degrees, against a range of clinical isolates of *Candida* and the toxin from K-500 was predominantly more active than that produced by the brewing isolate K-LKB. Under the conditions of the assay it would appear, therefore, that the two strains produce toxins which are biochemically distinct and exert their action against different sensitive strains. This confirmed the findings of earlier work (see section 3.3).

Similar patterns of sensitivity of the isolates to toxin action were apparent between the initial, and expanded, screens. However, with an increase in the number of isolates tested, the relationship between their sensitivity and their physiological niche became less pronounced. It was possible that the sensitivity to toxin action is a species-specific phenomenon. The isolates obtained from Ninewells Hospital for this screen were classified only as belonging to the genus *Candida*, with the exception of the two isolates from blood which were positively identified as *C.pseudotropicalis* and *C.parapsilosis*. Attempts were made to identify the clinical isolates to enhance investigations into the relationship between toxin action and isolate sensitivity. 74% of the clinical samples were identified using a commercial diagnostic kit and of these 86% were found to be strains of *C.albicans*.

Table 3.8 - Table showing the combined percentages of the predominant species of *Candida* found at various sites of the body (data collated from numerous surveys conducted in Europe and the U.S.A between 1962 and 1987, (Odds,1988)).

Candida Strains	Oral Cavity	G.I.T[a]	Anorectal	Vaginal (unselected groups)[b]	Vaginal (with local diseases)[c]	Blood	Urine
C.albicans	69.6	56.5	50.9	69.7	84.2	50.4	54.4
C.glabrata	6.6	16.1	9.1	11.7	5.5	9.7	20.2
C.guilliermondii	0.4	0.5	0.7	0.5	0.5	0.9	1.9
C.kefyr	1.0	0.7	0.1	0.4	0.4	0.2	0.2
C.krusei	1.7	2.6	2.9	2.6	1.7	1.1	2.1
C.parapsilosis	1.9	6.1	5.4	1.9	1.2	12.1	4.1
C.tropicalis	6.9	9.7	2.3	4.7	5.3	18.5	8.9
Other Candida	7.0	1.4	7.1	2.1	0.5	2.0	2.7
Unidentified	4.5	6.4	22.0	6.3	0.7	4.9	4.5

a - yeasts isolated from the gastrointestinal tract.
b - Distribution of species among yeasts isolated from the vagina of subjects without vaginitis, or presence or absence was not recorded.
c - Distribution of species among yeasts isolated from the vagina of subjects exclusively with signs and symptoms of vaginitis.

Table 3.9 - Table showing the frequencies of yeasts and/or *C.albicans* isolated from different sites in various types of subject (table collated from data presented by Odds, 1988).

Site of Isolation	Status of Subject	Yeasts Recovered (% range)	C.albicans Recovered (% range)
Oral Cavity	Healthy Patient	2.0 - 71.3 12.7 - 76.2	1.9 - 62.3 6.0 - 69.6
Anorectal	Healthy Patient	8.0 - 60.0 5.7 - 83.1	8.0 - 20.0 1.0 - 53.1
Vaginal	Healthy Patient	4.3 - 27.3 7.8 - 76.2	2.2 - 68.0 4.5 - 60.0
Skin	Healthy Patient	0.0 - 44.0 4.7 - 63.6	0.0 - 17.4 0.4 - 25.0
Outer Eye Surface	Healthy Patient	3.9 - 20.6 3.8 - 16.9	0.0 - 2.3 0.0 - 5.4
Urine	Patient	1.4 - 60.0	0.0 - 21.1

The proliferation of various species of *Candida* in the body is dependent not only on the site of isolation (Table 3.8) but also on the status of the individual *i.e.* whether they are healthy or predisposed to increased yeast invasion (Table 3.9). As Table 3.8 shows *C.albicans* is by far the most proliferate yeast found. In general it accounts for 60-80% of oral isolates with *C.glabrata* and *C.tropicalis* being found in only low frequency. In vaginal isolates there is a higher occurrence of *C.albicans* in patients with known vaginal pathologies (84%) than surveys of unselected groups (69%). This increase over the normal vestigial flora is further highlighted in Table 3.9. The percentage of *C.albicans* recovered from the vagina of healthy individuals was 11% compared with 84% in those showing signs of local disease. In blood cultures isolation of *C.albicans* is rather lower at 50%. This is close to the prevalence found in the stomach, intestine and faeces which is compatible with the suggestion that the gut is the portal entry for *C.albicans* into the bloodstream (Young, 1987). *C.tropicalis* and *C.parapsilosis* have a heightened incidence in blood cultures which implies a selection process in their favour over other *Candida* spp. in visceral sites. Yeasts can enter the urine from the blood stream or the urethra and the distribution pattern of *Candida* closely resembles that seen in the gut.

A differential sensitivity to the action of K-500 and K-LKB killer toxins was found amongst isolates of the same species type (section 3.4). Some strains were found to be resistant to toxin action whereas growth of others was completely inhibited. Of the two strains identified as *C.glabrata* one was resistant and the other showed 95% reduction in growth. Grouping of the isolates with respect to their response to the killer toxin of *W.mrakii* (K-500) could be used to biotype the *Candida* strains, as previously reported by Polonelli *et al.* (1983) and other opportunistic yeast isolates including *C.glabrata*, *C.parapsilosis* and *Cryptococcus neoformans* (Morace *et al.*, 1984; Caprilli *et al.*, 1985).

The patterns of sensitivity observed were likely to be as a result of a number of factors, including;

(a) the site of isolation, which will undoubtedly influence the species and strain type which is found.

(b) micro-environmental conditions within the niche, which may produce morphological changes in the isolates, thus, affecting their sensitivity to toxin action.
(c) the status of the individual which may also influence isolate type and cell wall characteristics and, therefore, their subsequent mode of action and inaction.
A combination of these factors will affect the interaction between the toxin molecules and their cell surface receptors. The type and number of receptors available may differ between isolates and their accessibility to toxin molecules may be influenced by any aberrations in cell envelope morphology.

3.7 Conclusions

Yeast strains of the ascomycetous genera of *Pichia* and *Williopsis* showed the broadest range of killer activity against a limited number of sensitive strains of *S.cerevisiae* and *Candida spp. W.mrakii* (K-500) consistently displayed the strongest interstrain lethality and extensive anti-*Candida* activity. As a consequence this killer yeast strain was selected to further investigate toxin production and toxin action against sensitive yeasts and attempts were made to determine the biochemical nature of the toxin produced.

No direct correlation was observed between isolate collection site and toxin sensitivity, however, the results highlighted the potential use of killer yeast and toxin preparations in the epidemiological typing of pathogenic yeast strains.

CHAPTER FOUR

Production of *Williopsis mrakii* Killer Toxin

4.1 Introduction

Killer toxin protein is known to be secreted during the exponential phase of growth in the K1 killer yeast *S.cerevisiae* (Young and Philliskirk, 1977; Palfree and Bussey, 1979). Growth-associated secretion of proteins from microbes will, therefore, be affected by biomass concentration (Grafe, 1982). Any factors which directly influence cell growth such as culture media composition, available carbon and nitrogen sources and oxygen and pH requirements, will subsequently affect the synthesis of microbial metabolites.

It was the aim of this area of research to investigate the growth of the killer yeast *W.mrakii* (K-500) in different media, and the effect of static, agitated and oxygenated propagation systems on toxin production. It was hoped, therefore, to maximise toxin production in large-scale fermentations. Attempts were made to partially purify the killer factor produced by ultrafiltration and dialysis.

4.2 The Effect of Media Composition on Toxin Production by the Killer Yeast *W.mrakii* (K-500)

A comparison was made of the effect of complex (YEPD), synthetic (SLM) and minimal (YNBGS) media on the growth of the killer yeast *W.mrakii* (K-500) and on toxin production (see section 2.7.1). Each media type was inoculated with 5 ml (10% inoculum) of a 24 hour starter culture. Cells were grown statically at 25^{o}C and samples were taken over a 48 hour period. Cell numbers were estimated (section 2.3) and the mean generation time, the time for one cell to divide into two during active growth, was calculated (section 2.7.1). The extent of toxin production was assessed by measurement of the killing activity of the culture supernatant against the indicator strain *C.glabrata* (S-388), using an agar diffusion well bioassay (section 2.6.2).

The results are presented in Figures 4.1 (YEPD), 4.2 (SLM) and 4.3 (YNBGS). All of the media investigated produced comparable growth and toxin production from the killer yeast *W.mrakii*. In all three, typical growth of the yeast was observed with cells entering a stationary phase after a period of 24 hours. However, growth of the killer yeast was best supported by the minimal medium YNBGS (mean generation time of 2.6

Figure 4.1 - Cell growth and toxin production by the killer yeast *W.mrakii* (K-500) in a complex medium, YEPD. Toxin production was monitored using an agar diffusion well bioassay.

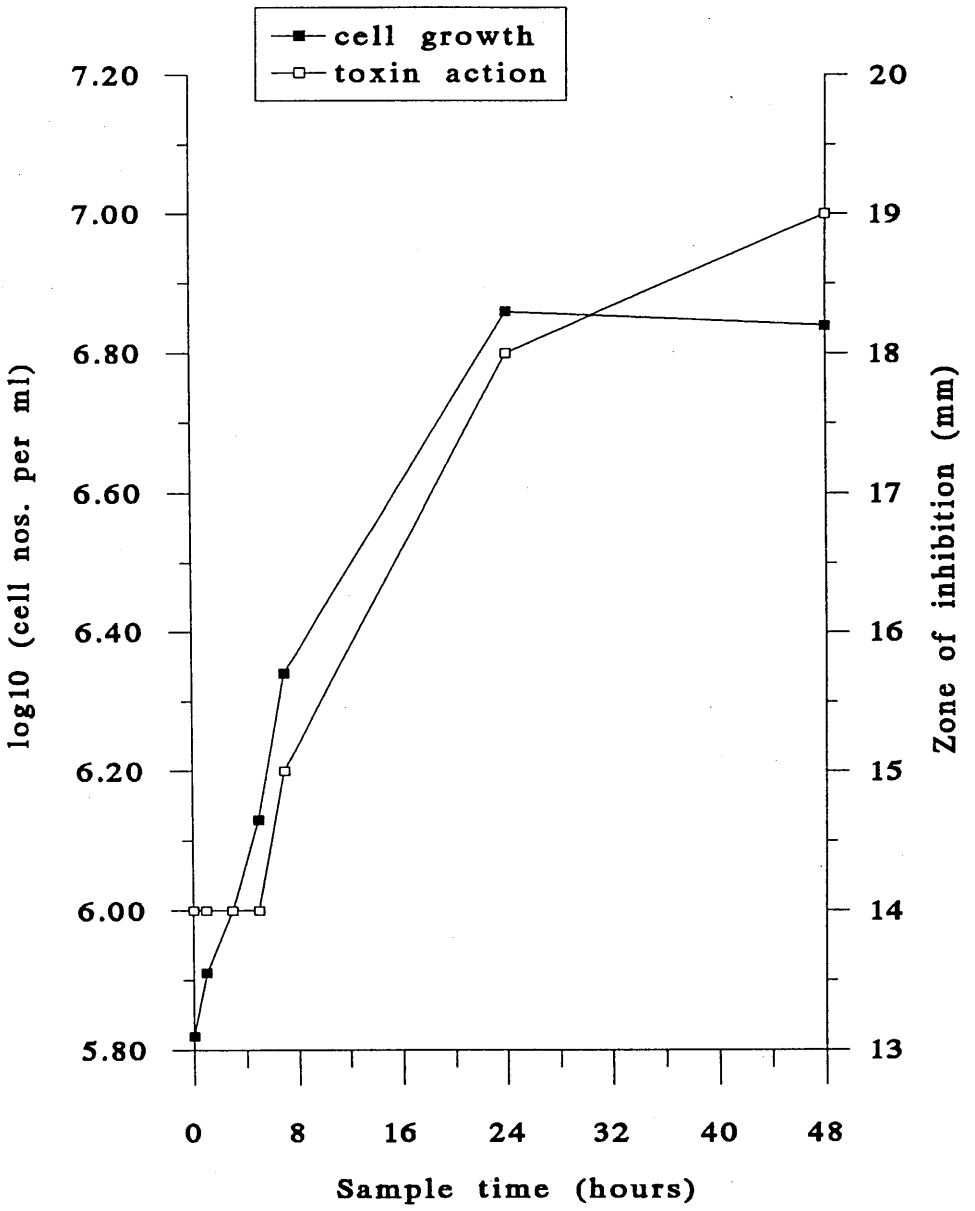


Figure 4.2 - Cell growth and toxin production by the killer yeast *W.mrakii* (K-500) in a synthetic medium, SLM. Toxin production was monitored using an agar diffusion well bioassay.

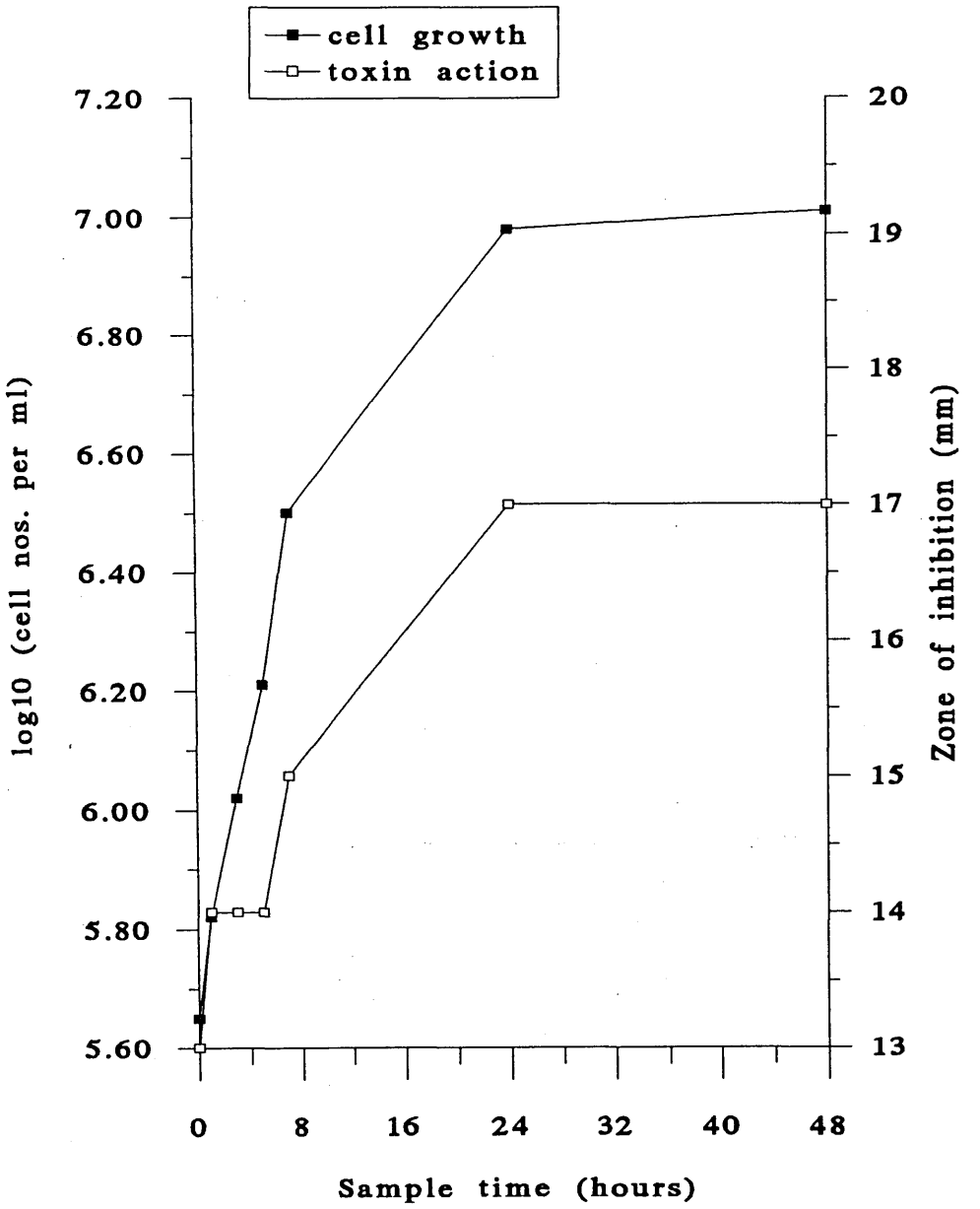
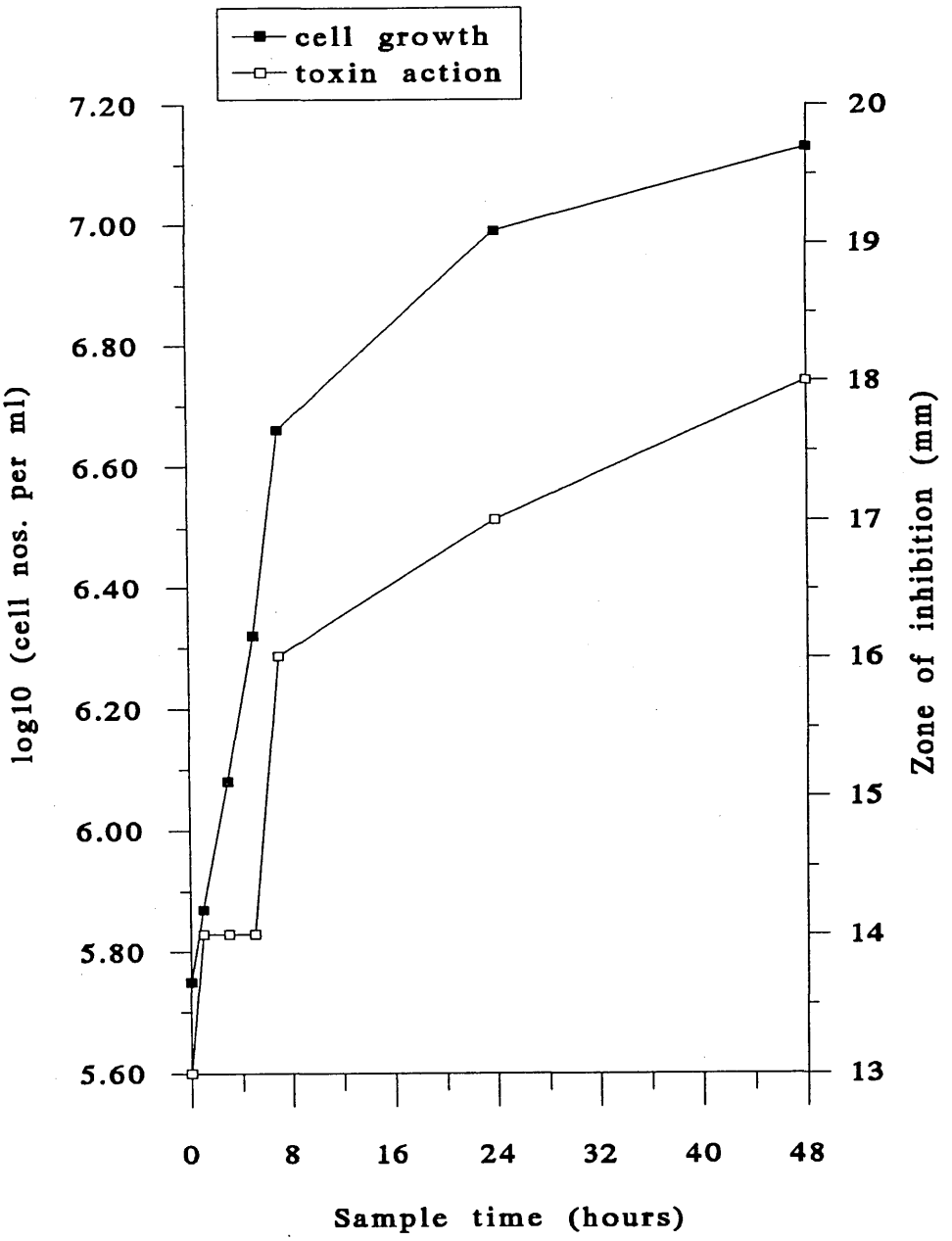


Figure 4.3 - Cell growth and toxin production by the killer yeast *W.mrakii* (K-500) in a minimal medium, YNBGS. Toxin production was monitored using an agar diffusion well bioassay.



hours), rather than by the richer media, SLM and YEPD (mean generation times of 3.1 hours and 4.9 hours respectively). Toxin activity, as measured by zones of inhibition in the agar diffusion bioassay, reflected cell growth. In each case, the killing activity of the toxin produced increased during the exponential phase of growth and plateaued when stationary phase was reached. The richer medium YEPD, produced killer factor with the greatest activity against the sensitive strain tested, whereas toxin produced in SLM and YNBGS was less active.

4.3 Toxin Production in *W.mrakii* (K-500) Under Static Culture Conditions

(a) Growth

Continued investigations into the production of killer toxin by the yeast *W.mrakii* (K-500) used the minimal medium, YNBGS, as described in section 2.7.1. The yeast was grown in 50 ml of the above media at 25^{0}C, and samples were removed aseptically to determine cell counts (section 2.3), protein content of the extracellular medium (section 2.4) and toxin activity, against the indicator strain *C.glabrata* (S-388), using a microtitre plate assay (section 2.6.3).

The culture broth was sampled at intervals over a period of 186 hours and the results are shown in Figures 4.4 and 4.5. From a loop-inoculum, it was observed that cells of K-500 progressed into exponential growth between 24 and 72 hours, and into stationary growth following an incubation of 90 hours. The mean generation time was calculated as 13.4 hours. Toxin production, in part, reflected cell growth, however, it appeared that the levels of the killer factor continued to increase as cells entered stationary phase. The increase of protein in the extracellular medium mirrored the cell growth of the killer yeast.

(b) Processing of Fermentation Media

Initial investigations had suggested that the killer toxin produced by *W.mrakii* (K-500) was a protein with a molecular mass greater than 16 kDa. Therefore, a scheme for downstream processing of the killer factor was designed to incorporate an ultrafiltration step which would concentrate material greater than 10 kDa in size.

Figure 4.4 - Cell growth and toxin production by the killer yeast *W.mrakii* (K-500) under static conditions. Toxin production was monitored against the sensitive strain *C.glabrata* (S-388), using a microtitre plate assay.

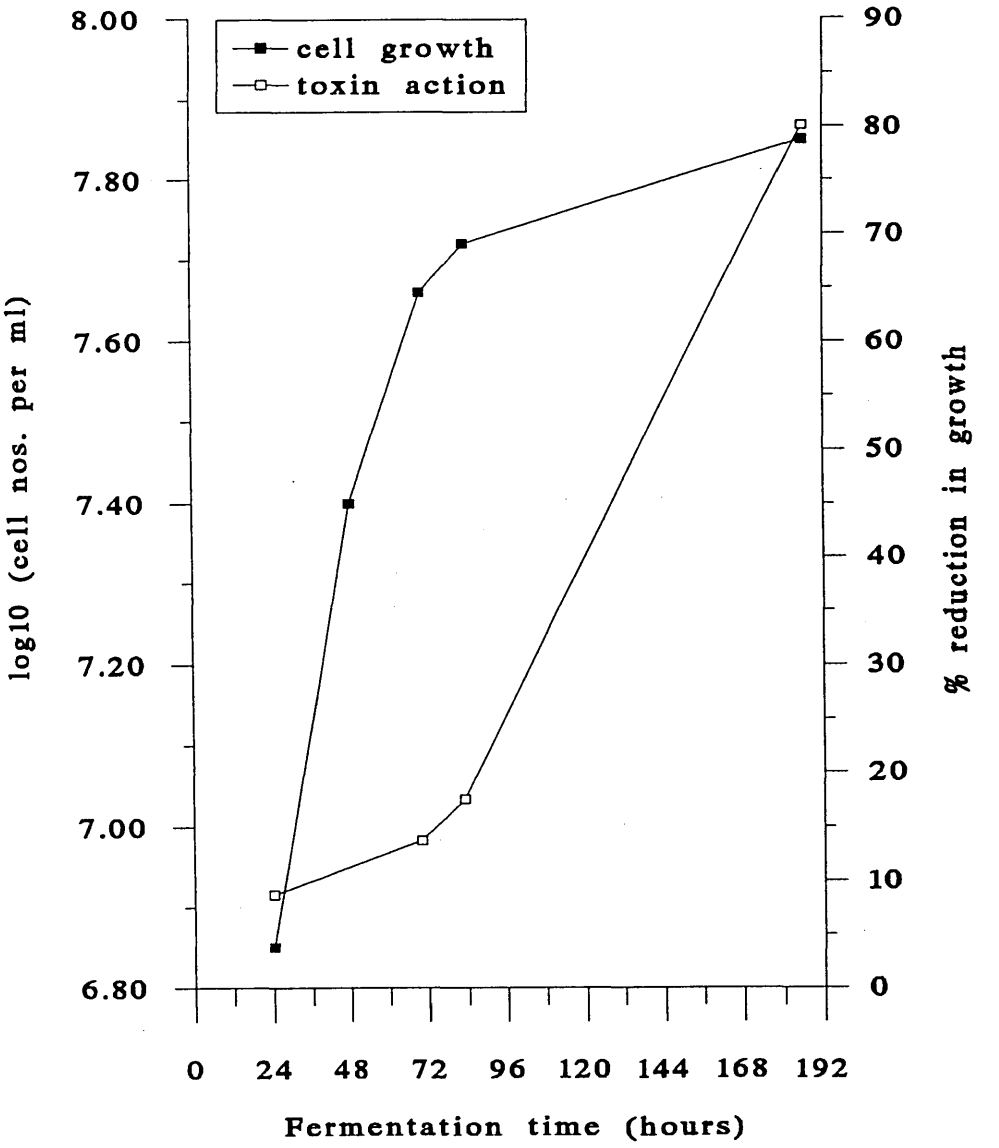
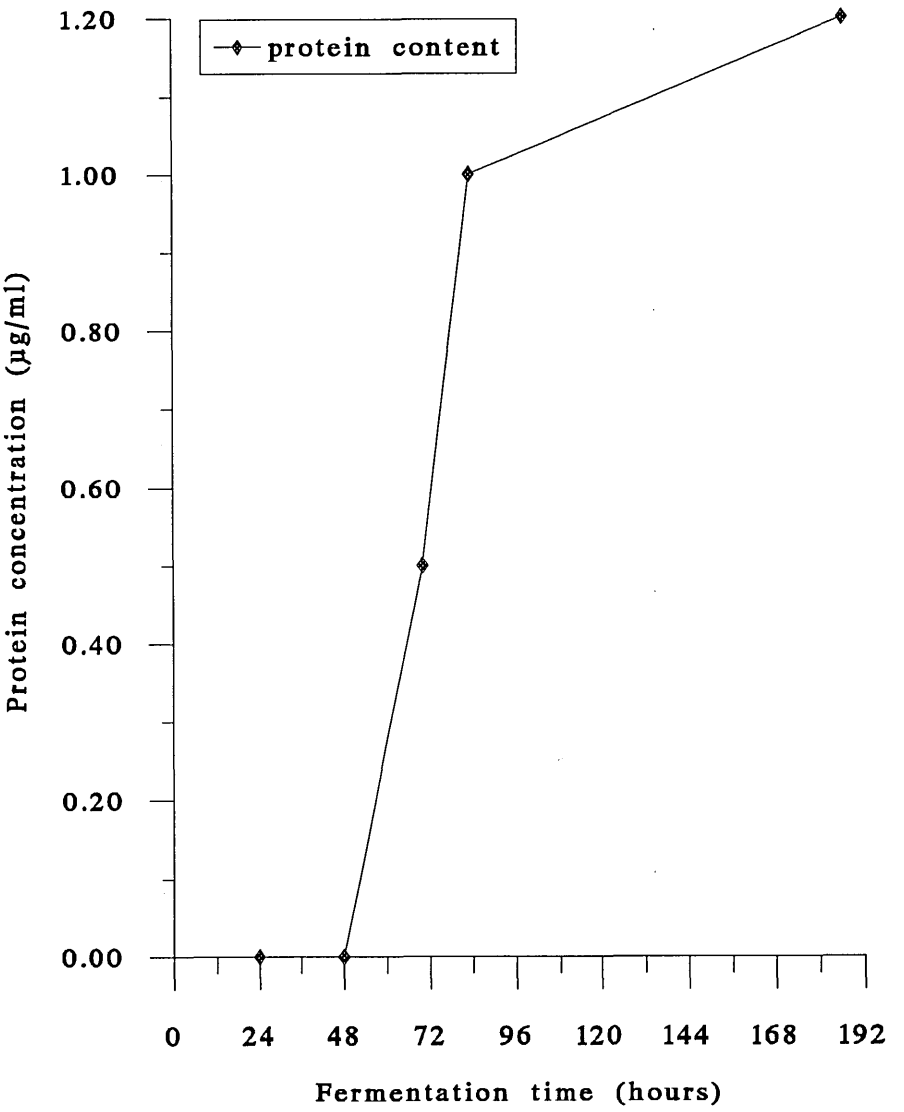


Figure 4.5 - Protein content of the extracellular media during growth of the killer yeast *W.mrakii* (K-500), under static conditions.



The downstream processing of cultures of *W.mrakii* is outlined in section 2.8.1. A 240 hour static culture (50 ml) of the killer yeast was centrifuged and filtered through a 0.45 μm membrane (S1), to remove cellular material. The cell-free supernatant was concentrated to 5 ml (S2) by Amicon stirred ultrafiltration using a PM10 membrane. The retentate was freeze-dried and a sample of the permeate (S3) retained for analysis. The lyophilisate was reconstituted at a concentration of 60 mg/ml dry weight (S4) and all samples were assayed against the indicator strain *C.glabrata* (S-388), using an agar diffusion bioassay. Results are shown in Table 4.1.

Extracellular medium from a culture of *W.mrakii* displayed killing activity against the sensitive strain *C.glabrata*, producing a zone of inhibition of 16.5 mm. Concentration of the cell-free supernatant by ultrafiltration yielded two fractions, the retentate (>10 kDa) and the permeate (<10 kDa). The retentate showed increased lethality (a zone of 19.0 mm) against S-388, whereas the permeate showed negligible activity. The retentate was freeze-dried and also tested for killing activity. Further concentration of the toxin activity at this stage was displayed by an increase in inhibition of the sensitive strain (a zone of 19.5 mm).

4.4 Effect of Oxygenation and Agitation on Toxin Production in *W.mrakii* (K-500)

It was hoped to gain an insight into the oxygen demand of the killer yeast *W.mrakii* (K-500) for subsequent growth and toxin production studies on a larger scale. Three fermenters were set up as described in section 2.7.2.1. Fermenter A was essentially anaerobic because of a continuous flow-through of nitrogen prior to inoculation. Fermenter B was continuously aerated at a rate of 200 cm^3/min and fermenter C was gently agitated. After inoculation, fermenter A was maintained under static conditions whilst fermenters B and C were continuously agitated. Each system was kept at a temperature of 25^{o}C, and samples were removed to determine cell numbers (section 2.3), protein content (section 2.4) and the toxin activity present in culture broths, using a microtitre assay (section 2.6.3).

The resultant cell growth and toxin activity observed in each fermenter is shown in Figures 4.6 to 4.8. The amount of dissolved oxygen available greatly influenced the

Table 4.1 - Toxin activity measured during the downstream processing of small, static cultures of *W.mrakii* (K-500). Samples S1 to S4 were assayed for activity against *C.glabrata* (S-388), using an agar diffusion bioassay.

Sample	Zones of Inhibition (mm)
Cell-free Supernatant (S1)	16.5
Ultrafiltration Retentate (>10 kDa) (S2)	19.0
Ultrafiltration Permeate (<10 kDa) (S3)	slight staining around well
Retentate Lyophilisate (60 mg/ml) (S4)	19.5

Figure 4.6 - The effect of '0%' dissolved oxygen (fermenter A) on the growth and toxin production in the killer yeast *W.mrakii* (K-500). Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.

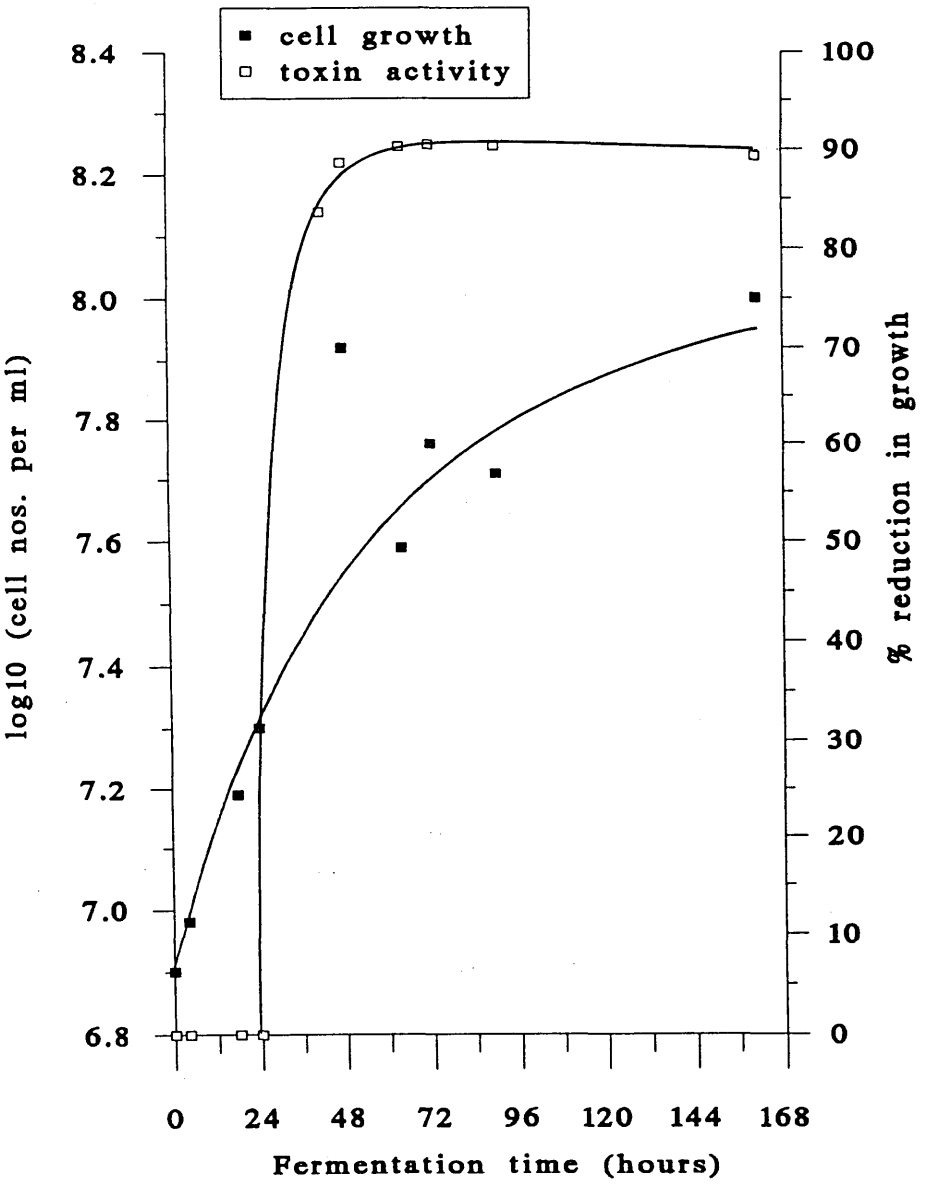


Figure 4.7 - The effect of direct aeration (fermenter B) on the growth and toxin production in the killer yeast *W.mrakii* (K-500). Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.

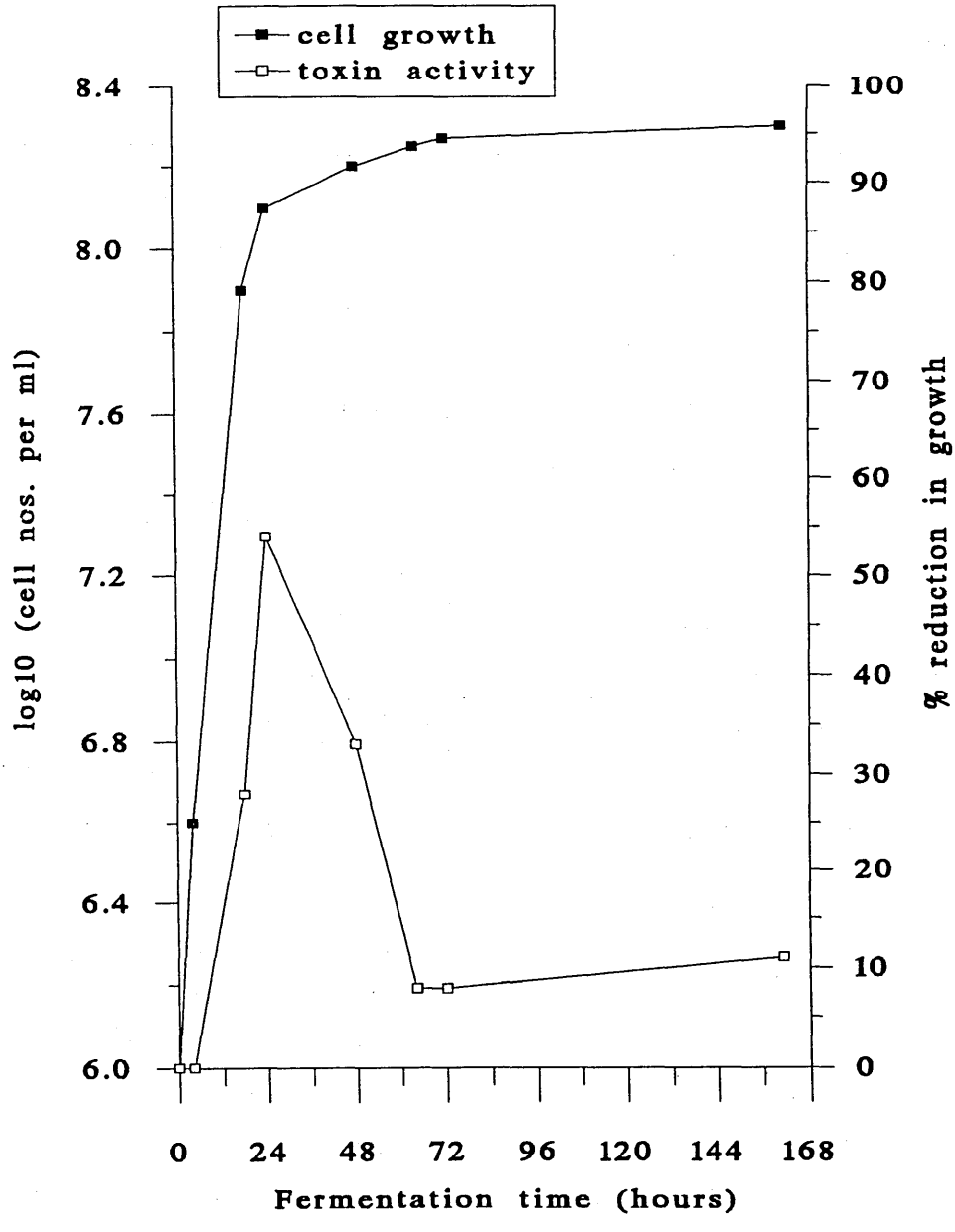
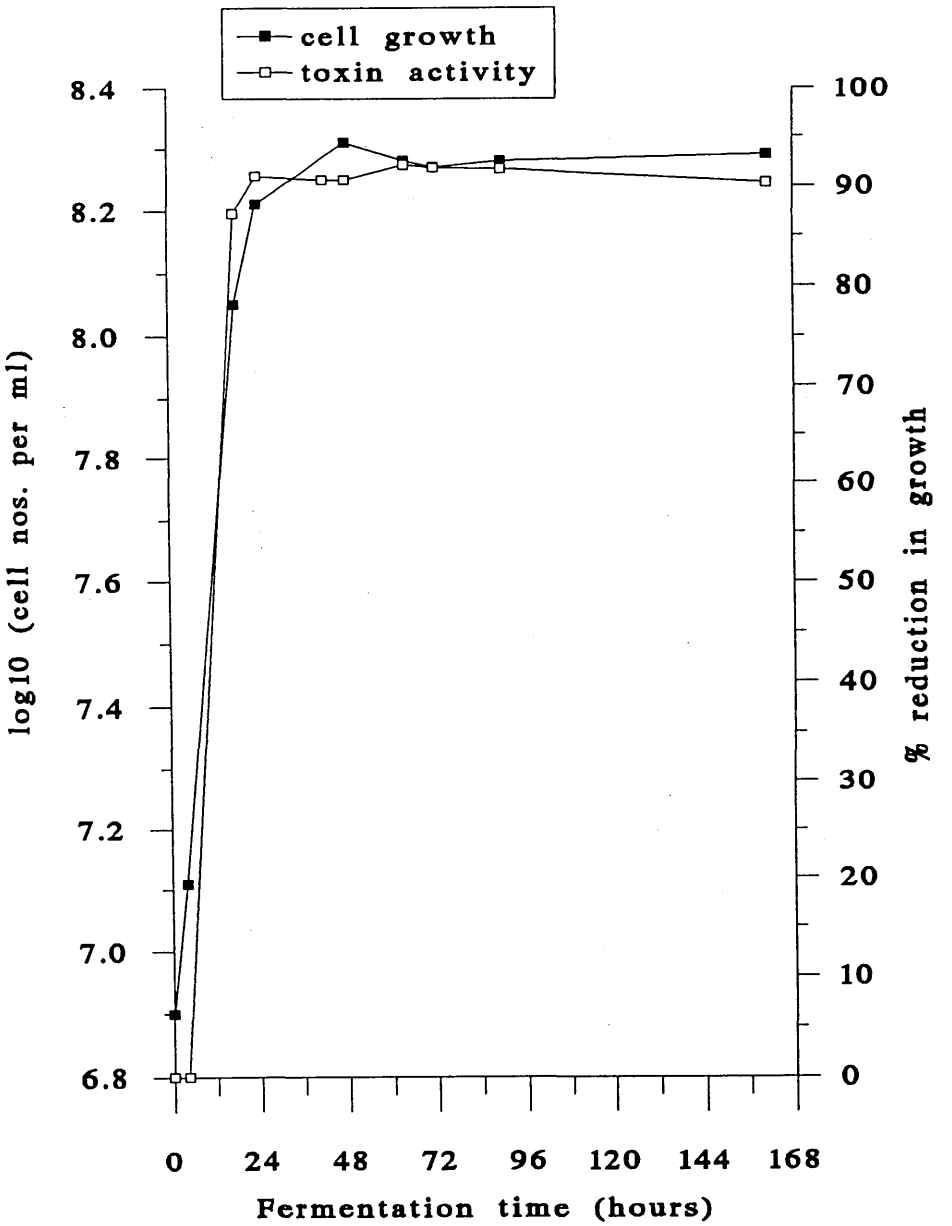


Figure 4.8 - The effect of agitation (fermenter C) on the growth and toxin production in the killer yeast *W.mrakii* (K-500). Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.



growth and division of the cells. In C (Figure 4.8), under gentle agitation, the mean generation time of the cells was 4.3 hours, in A (anaerobic) it was 10.4 hours (Figure 4.6) and in B (aerated) 4.0 hours (Figure 4.7). The maximum cell growth achieved was reduced in the anaerobic and aerated cultures in comparison to fermenter C.

Cell growth can be closely correlated to production of an active toxin. In each case, toxin activity increased markedly during the exponential phase of growth of the cells and the maximum toxin activity for the volume assayed (100 µl) was reached at the same time as cells progressed into stationary phase.

As a more critical assay of toxin activity, reduced volumes (100 to 10 µl) of cell-free supernatants from the 4, 24, 64 and 160 hour samples, were assessed for their killing activity against the indicator strain *C.glabrata* (S-388). From these'sensitivity profiles' (similar to those described in section 6.2) an estimate of the number of units of toxin activity present in each sample was possible (data not shown). One unit of activity was defined as the volume of cell-free supernatant which produced approximately 100% reduction in the growth of the sensitive strain. By calculation the number of units present in each culture (total volume 900 ml) could be made.

In fermenter C (Figure 4.11) the levels of toxin in the culture appeared to mirror production of protein by the killer yeast under these conditions. Beyond 72 hours, when cells had reached mid to late stationary phase, no further increase in toxin activity was observed. At this stage there was only a small increase in the concentration of protein detected (4.0 to 5.0 µg/ml). In fermenter A (Figure 4.9), the appearance of high levels of protein was coincident with only a very small increase in toxin activity. No further toxin appeared to be produced by the cells after mid-stationary phase was reached yet the protein in the culture increased from 6.8 to 10.4 µg/ml. In fermenter B (Figure 4.10), toxin activity was only 50% of that seen in fermenters A and C. In the latter, this activity was maintained throughout the fermentation but in fermenter B it fell to minimum levels after 72 hours. In the aerated culture the the concentration of protein present in the extracellular medium rose to a maximum level of 8.2 µg/ml at 64 hours and fell steadily to 6.2 µg/ml by the end of fermentation.

Figure 4.9 - The effect of '0%' dissolved oxygen on protein levels during growth of the killer yeast *W.mrakii* (K-500) and its relationship to toxin production in fermenter A. Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.

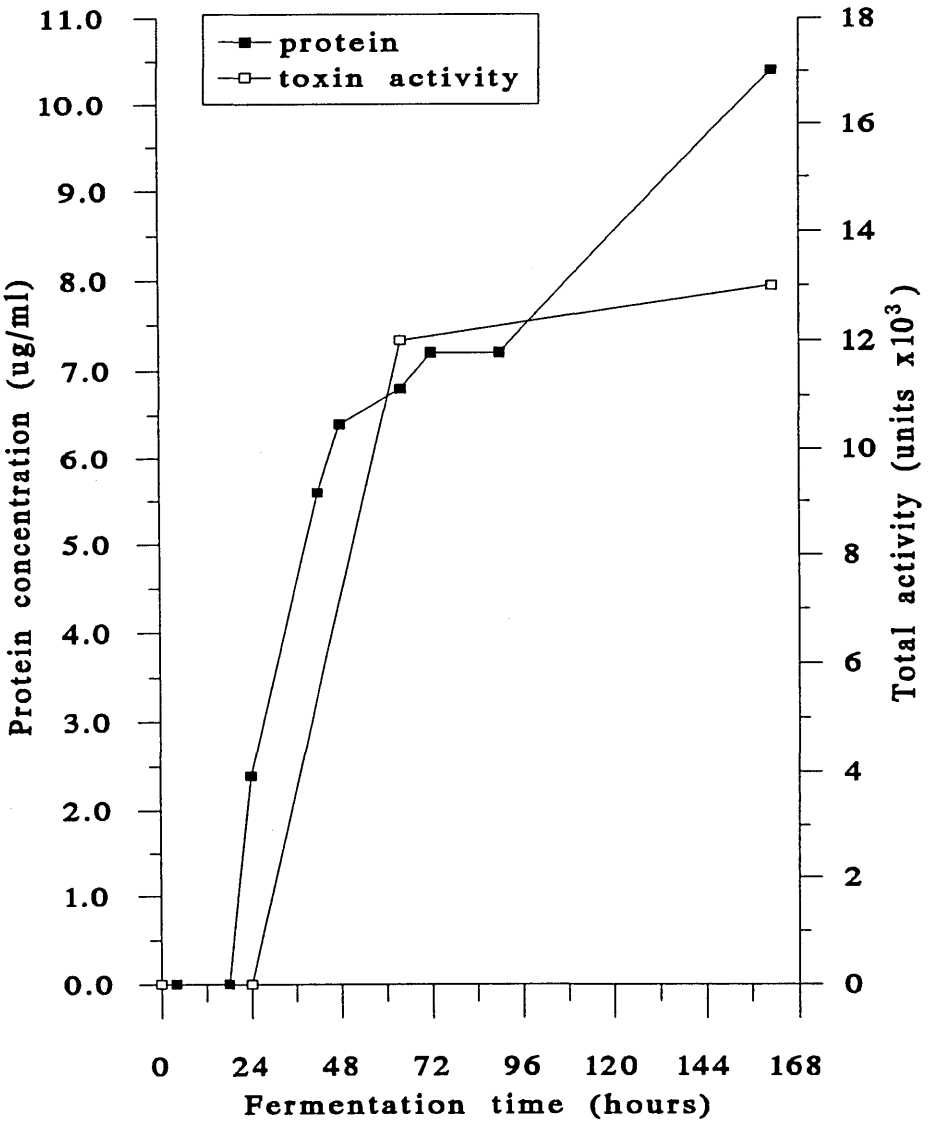


Figure 4.10 - The effect of direct aeration on protein levels during growth of the killer yeast *W.mrakii* (K-500) and its relationship to toxin production in fermenter B. Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.

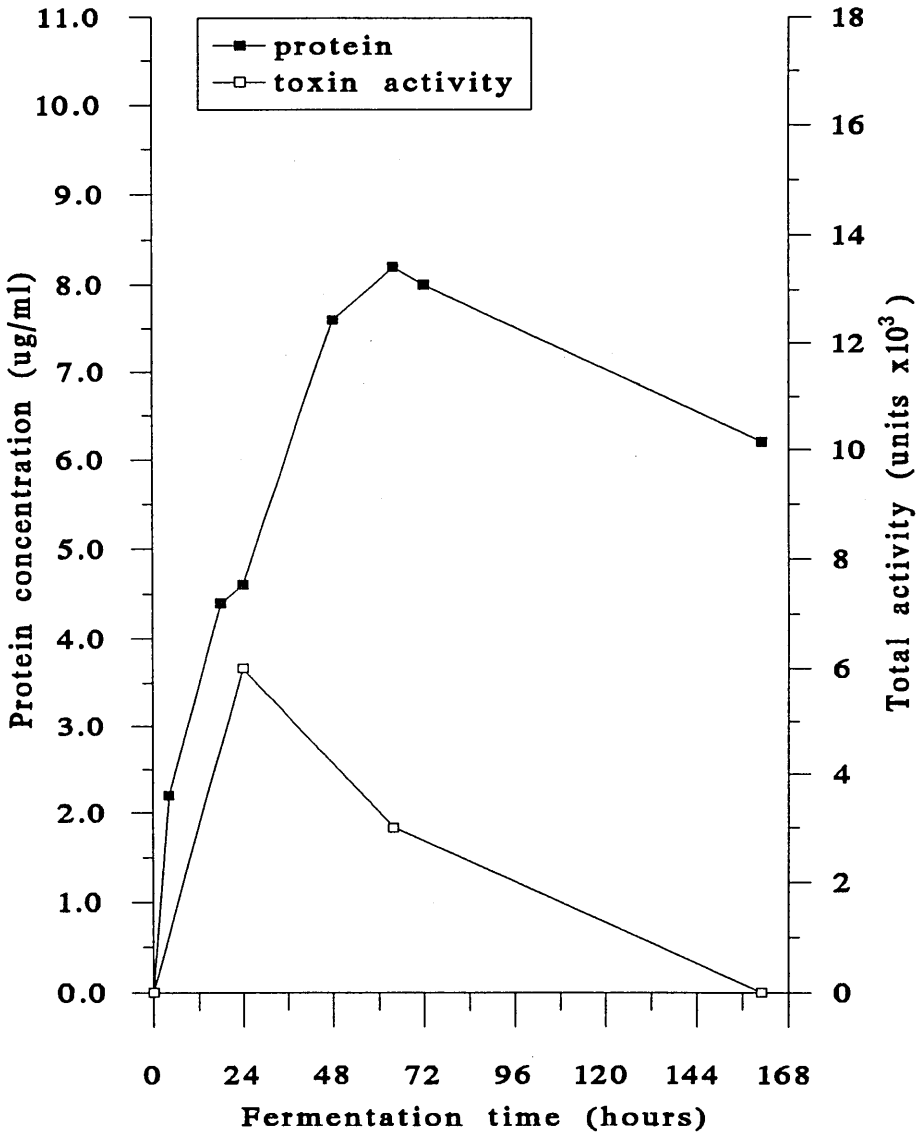
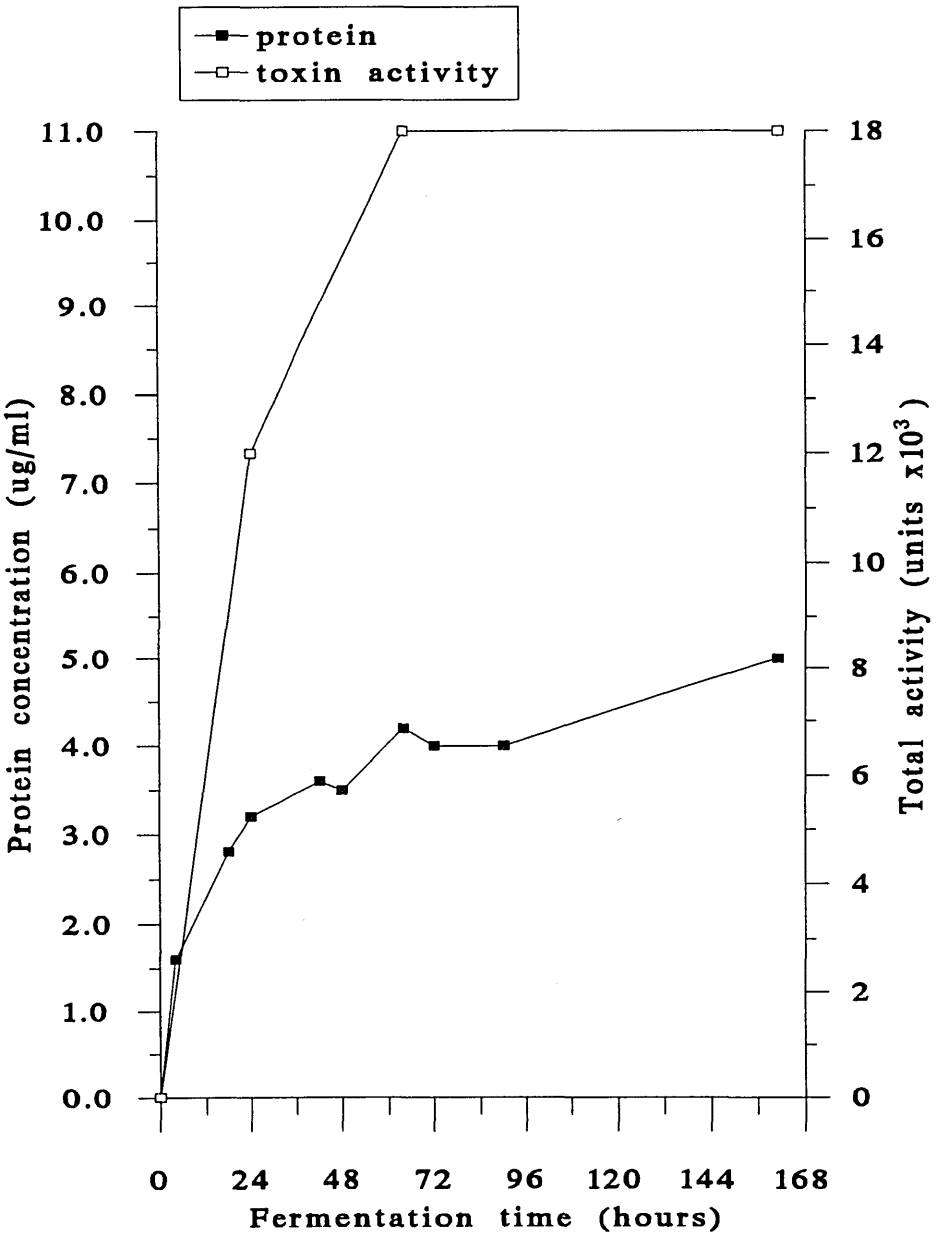


Figure 4.11 - The effect of agitation on protein levels during growth of the killer yeast *W.mrakii* (K-500) and its relationship to toxin production in fermenter C. Toxin activity in a fixed volume of cell-free supernatant (100 µl) was measured against *C.glabrata* (S-388), using a microtitre assay.



4.5 Small-Scale Fermentations of the Killer Yeast *W.mrakii* (K-500)

Fermentation with the killer yeast was carried out in the minimal medium YNBGS (section 2.7.1), over a three day period. Overnight seed cultures (100 ml) were used to inoculate two one litre fermenters containing 800 ml of fresh media (Figure 4.12). Cultures were incubated at 25^{o}C with gentle agitation and then processed as outlined above.

At completion, cell growth in both fermenters was comparable, $8.87x10^{7}$ and $1.03x10^{8}$ cells/ml respectively and, toxin activity and protein levels in the extracellular media were very similar ($S1_{A}$ and $S2_{B}$ in Table 4.2). The two cultures were combined and cellular material was removed by continuous centrifugation and microfiltration. The working volume at this stage was reduced from 1.6 to 1.2 litres. The protein content of the cell-free supernatant (S2), was approximately half (0.70 µg/ml) of that observed in the initial cultures (an average of 1.55 µg/ml) and showed reduced killing activity (25% less) in the microtitre assay. The supernatant was then concentrated by ultrafiltration. Samples of both the permeate, material less than 10 kDa (S3), and the retentate, material greater than 10 kDa (S4), were assayed for protein and toxin activity. Negligible amounts of protein were detected in the permeate and it showed no lethality against the indicator strain in a microtitre assay. The retentate contained 1.60 µg/ml of protein, only 22% of that expected from a 6-fold concentration of the cell-free supernatant (S2). In the concentrate, one unit of toxin activity was equivalent to 70 µl of the sample which indicated an 80% loss of killing activity at this stage. The retentate was further concentrated (20-fold) by freeze-drying and reconstituting of the lyophilisate in a reduced volume of distilled water. This process resulted in a slight loss of protein (5%) and further reduction in the lethality of the toxin preparation (42%).

Figure 4.12 - Flow-diagram showing the fermentation of *W.mrakii* (K-500) killer yeast and subsequent downstream processing of the killer factor. Samples $S1_A$ to S5 were removed for assessment of toxin activity and protein content (see Table 4.2).

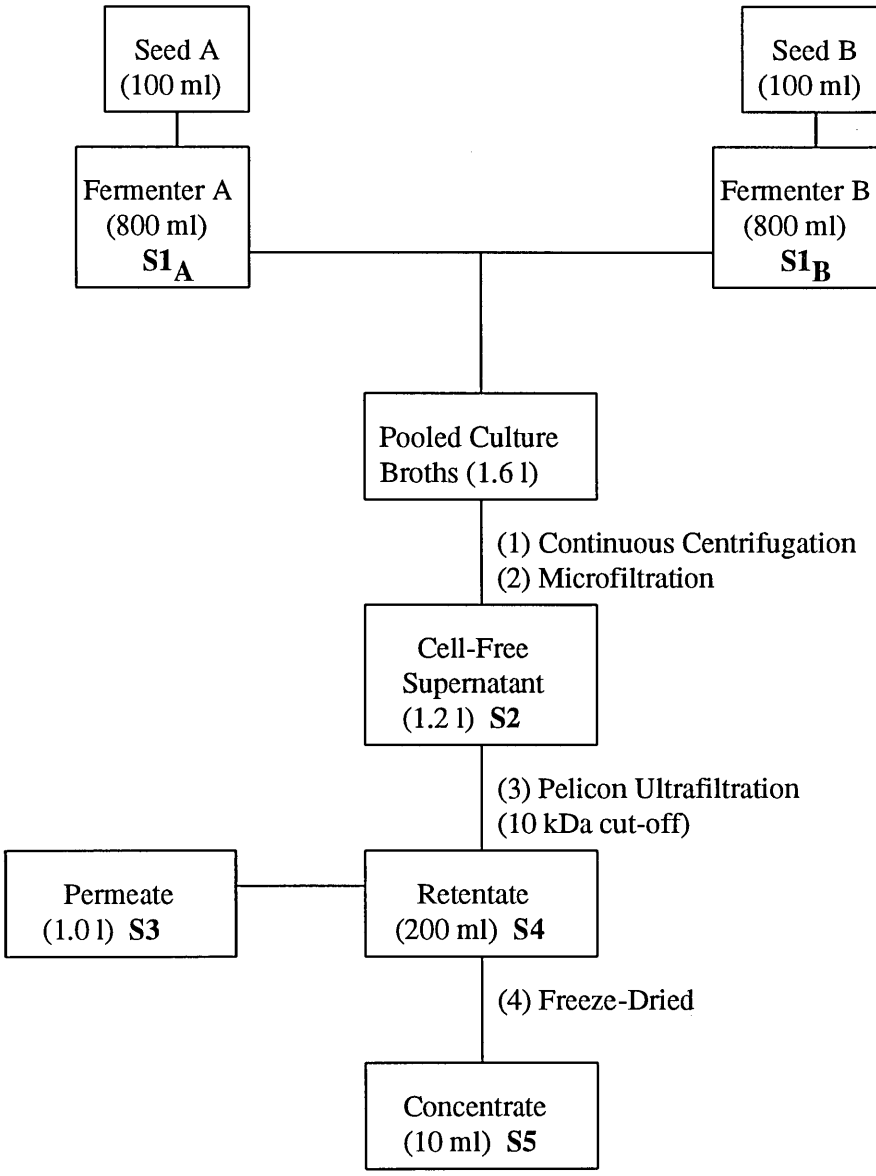


Table 4.2 - Table showing the maintenance of *W.mrakii* (K-500) killer toxin activity during downstream processing. At each stage of the fermentation and processing of the extracellular medium (see Figure 4.12), samples were assayed for protein content and toxin activity against *C.glabrata* (S-388), using a microtitre assay.

Sample	Volume of Toxin Producing 100% Growth Reduction (= 1 Unit of Activity)	Volume of Sample (ml)	Total Activity (units)	Loss of Activity (%) [a]	Protein Content (µg/ml)	Total Protein (mg)	Loss of Protein (%) [a]	Specific Activity (units/mg)
$S1_A$	60	800	13333	0	1.30	1.04	0	12820
$S1_B$	60	800	13333	0	1.80	1.44	0	9259
S2	80	1200	15000	25	0.70	0.84	66	17857
S3	0	1000	0	100	0	0	100	0
S4	70	200	2857	80	1.60	0.32	76	8928
S5	6	10	1666	42	30.20	0.302	5	5517

a - the percentage losses in toxin activity and protein content at each stage were calculated with respect to the previous sample

4.6 Large-Scale Fermentations of the Killer Yeast *W.mrakii* (K-500)

In order to increase the amount of toxin preparation available for future studies it was proposed to perform two large-scale (40 litre) fermentations of the killer yeast. The fermentations were carried out under the guidance of the Fermentation Section of the Microbiology Division of Glaxo Group Research.

Fermentation 1

Based on earlier studies, the parameters for the initial fermentation were decided as outlined in Figure 4.13. *W.mrakii* killer yeast was grown at 25^{o}C in the minimal medium YNBGS and the culture was gently agitated during fermentation. In light of the studies reported in section 4.4, there was no direct aeration of the culture and nitrogen was passed over the base seal to prevent contamination. Several samples were taken over the first 24 hour period to monitor cell growth by absorbance measurements at 600 nm.

Under the above conditions there was very poor growth of the yeast over 24 hours, an absorbance of 0.1 was measured compared to 0.5 in a static culture initiated at the same time, therefore, the fermentation was prolonged for a further 24 hours. Cell growth increased with the change in culture conditions, *i.e.* aeration of 1.5 l/min, but the cell density of 3.67×10^{7} cells/ml was only approximately 40% of that expected. The toxin-containing extracellular medium was harvested and processed, as described in section 2.8.2, to yield 59.87 g of freeze-dried material. Samples were taken for assessment of killing activity against a sensitive strain of *C.albicans* (C316), using a microtitre assay (section 2.6.3). The toxin activity was compared to that observed in a static fermentation of the killer yeast which was run simultaneously (Table 4.3).

Unconcentrated samples (1-3), obtained during large-scale toxin production displayed no killing activity against the sensitive strain C316 (data not shown). The maximum toxin activity achieved was an 85% reduction in growth of C316. This was detected in the 24 hour static culture of the killer yeast when concentrated 10-fold by freeze-drying (sample 4). No increase in killing activity against this strain was observed in the 48 hour sample (sample 6). However, a marked increase (28 to 83% growth reduction) was

Figure 4.13 - Flow-diagram showing the procedure followed during the first large-scale (40 litre) fermentation of the killer yeast *W.mrakii* (K-500).

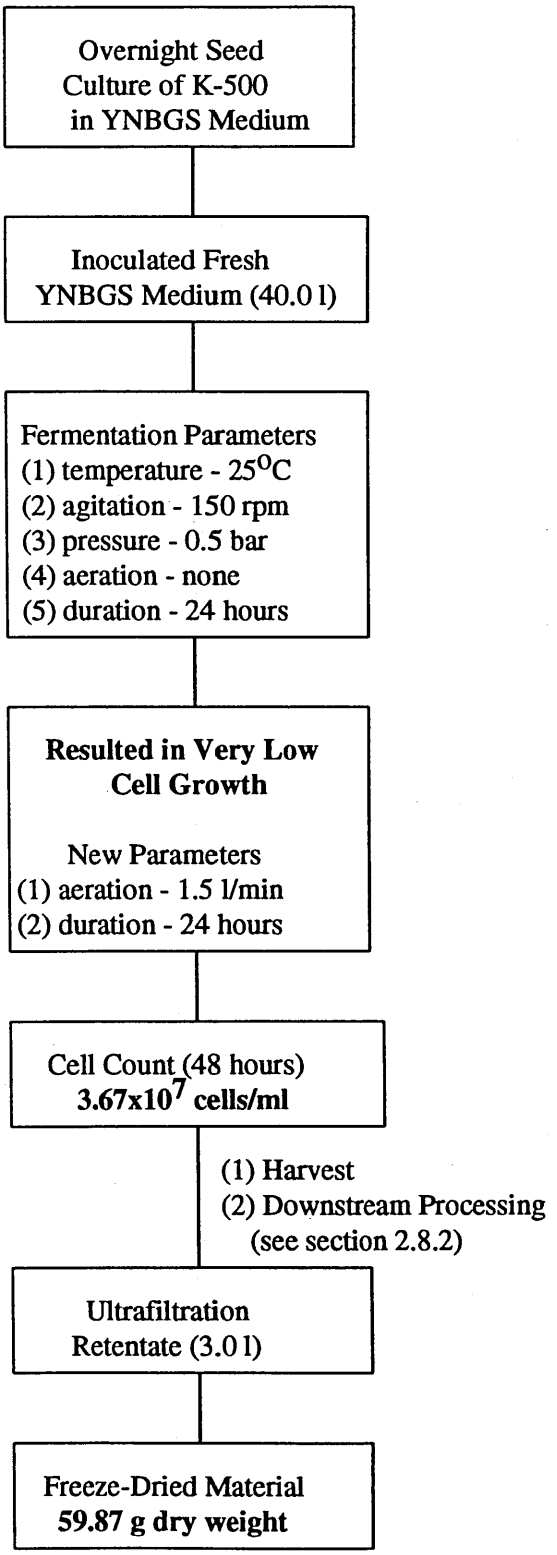


Table 4.3 - Toxin activity during the first large-scale fermentation and processing of the killer yeast *W.mrakii* (K-500). Activity was assessed against a sensitive strain of *C.albicans* (C316), using a microtitre assay. Results were compared to a static fermentation of the same yeast.

Samples assayed:

(1) cell-free supernatant from 48 hour large-scale fermentation[1]

(2) microfiltrate from large-scale fermentation[1]

(3) permeate (<10 kDa) from large-scale fermentation[1]

(4) 10-fold concentrate from 24 hour static fermentation

(5) 10-fold concentrate from 24 hour large-scale fermentation

(6) 10-fold concentrate from 48 hour static fermentation

(7) 10-fold concentrate from 48 hour large-scale fermentation

(8) lyophilised retentate (>10 kDa), 500 mg/ml dry weight, from 48 hour large-scale fermentation

Volume of Sample Assayed (µl)	Percentage Reduction in Growth of C316 Produced by Each Sample				
	4	5	6	7	8
100	84.6	27.9	84.3	78.8	79.2
75	74.3	0	84.0	79.6	80.3
50	0	0	85.1	81.8	82.1
25	0	0	67.8	59.5	19.7
10	0	0	0	0	0

1 - unconcentrated samples 1, 2 and 3 did not show significant killing activity against the sensitive strain C316.

Table 4.4 - Protein and carbohydrate analysis of the lyophilisate from the initial large-scale fermentation of *W.mrakii* (K-500).

Analysis	Concentration in lyophilisate (mg/ml)	Total Yield (g)	Percentage of Dry Weight (%)
Protein	0.032	0.00383	0.06
Carbohydrate	383	45.86	76.6

Lyophilisate was reconstituted at a concentration of 500 mg/ml dry weight prior to analysis

Table 4.5 - Protein and carbohydrate analysis of samples before and after dialysis. Lyophilisate was reconstituted at a concentration of 500 mg/ml and dialysed against distilled water. Dialysates were recovered for protein and carbohydrate analysis.

Sample	Protein Content (µg/ml)	Carbohydrate Content (mg/ml)
Lyophilisate (before dialysis)	47.2	383.0
Dialysate	13.6	3.2
Dialysis Tubing Washings	negligible	2.5
Dialysing Solution	negligible	260.0

observed between the 24 and 48 hour samples removed from the large fermentation (samples 5 and 7). Freeze-dried material (sample 8) was not significantly more active, even at lower assay volumes, despite the large concentration step involved.
Sample 8 was also assayed for protein (section 2.4) and carbohydrate content (section 2.5). Lyophilisate reconstituted at a concentration of 500 mg/ml contained 32 μg/ml protein and 383 mg/ml of carbohydrate. Table 4.4 shows the total yield of protein and carbohydrate in the lyophilisate.

In view of the large amounts of media carbohydrate remaining in the lyophilisate (76% of the final dry weight), attempts were made to dialyse the preparation to remove unwanted material. The lyophilisate was reconstituted at a concentration of 500 mg/ml and dialysed against distilled water at $4^{o}C$ for 19 hours (section 2.8.2.1). At completion the tubing was washed with distilled water and the dialysates recovered. Protein (section 2.4) and carbohydrate (section 2.5) assays were conducted on the preparation, before and after dialysis, on the tube washings and the dialysing solution.
The results in Table 4.5 demonstrated that dialysis resulted in the loss of 99% of the carbohydrate and 70% of the protein present in the preparation. Levels of protein in the dialysing solution were presumably too low for detection by the assay, however, the majority of the carbohydrate removed was detected.
The toxin activity present in each sample was measured using a microtitre assay. No lethality against the indicator strain *C.albicans* (C316) was detected in the dialysate, washings or dialysing solution (data not shown).

Fermentation 2

Low toxin activities in the initial fermentation were attributed to poor cell growth of the killer yeast. In an attempt to overcome this problem aeration of the culture was increased to 3.5 l/min . All other fermentation parameters remained unchanged and the procedure outlined in Figure 4.14 was followed. Samples were taken over 48 hours for cell counts (section 2.3) and protein analysis (section 2.4).

Good cell growth ($1.25x10^{8}$ cells/ml), when compared to earlier small-scale studies, was produced under the above conditions and the profile in Figure 4.15 demonstrated

Figure 4.14 - Flow-diagram showing the procedure followed during the second large-scale (40 litre) fermentation of the killer yeast *W.mrakii* (K-500).

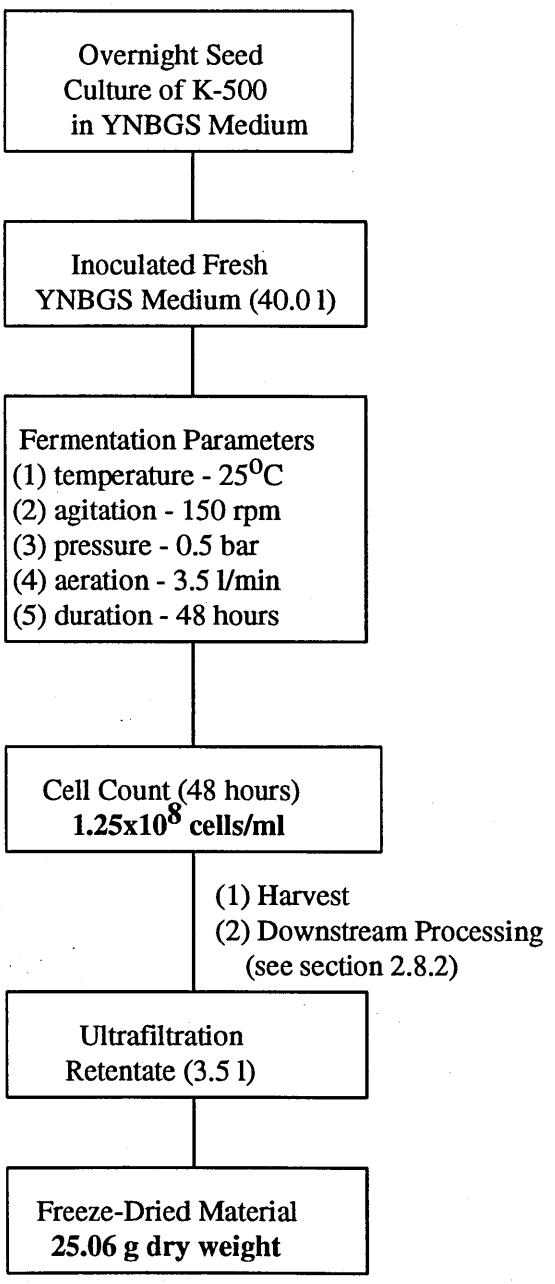
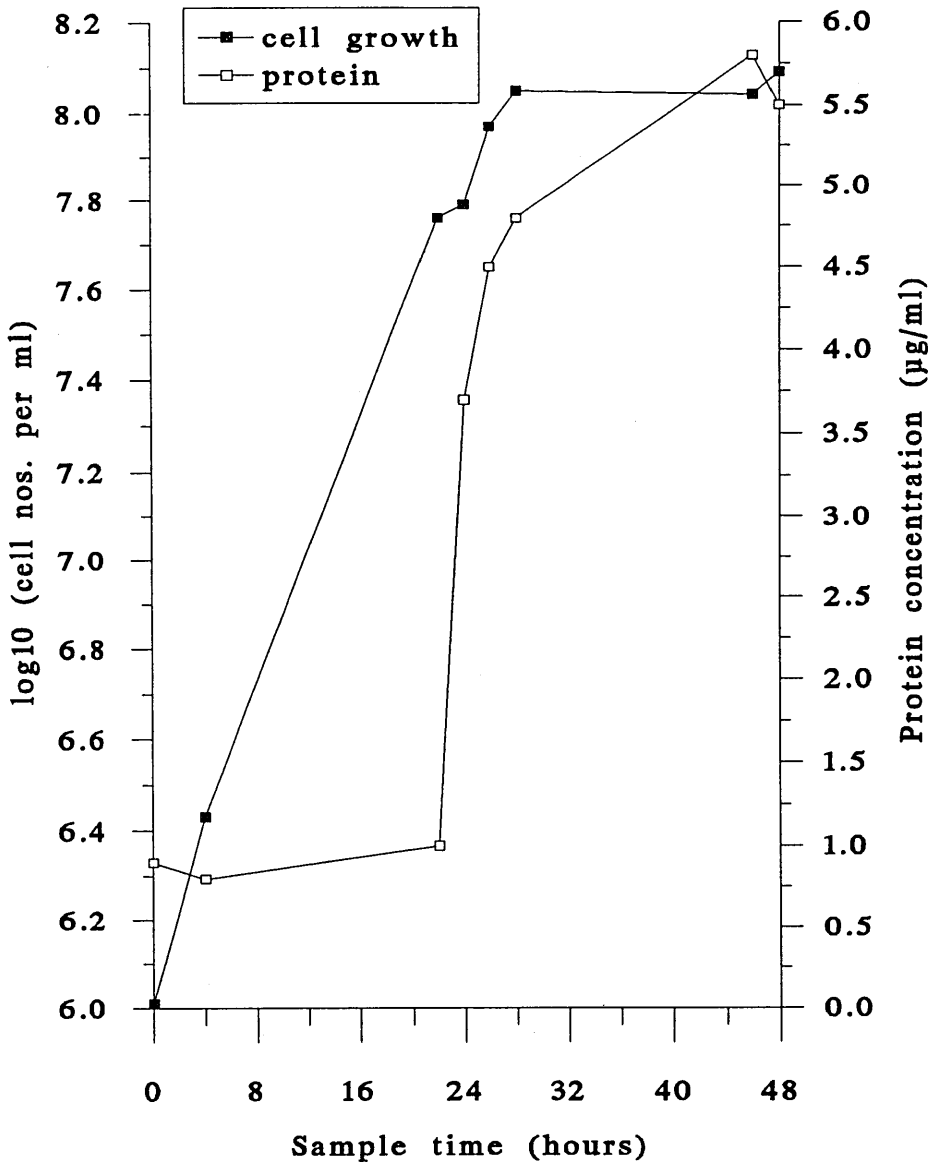


Figure 4.15 - Cell growth of the killer yeast *W.mrakii* (K-500) and protein levels in 1.0 ml of the extracellular medium during the second large-scale fermentation.



that the killer yeast *W.mrakii* showed typical growth characteristics. Cells grew exponentially over a 28 hour period before entering a stationary phase. The mean generation time was calculated as 4.07 hours. Low residual levels of protein (>1.0 μg/ml) were present in the fermentation media at inoculation and did not increase appreciably over the first 22 hours. Within 4 hours the levels of protein present in the extracellular medium increased considerably to a concentration of 4.5 μg/ml. As cells entered stationary phase there was a further detectable increase to 5.8 μg/ml before the levels fell to 5.5 μg/ml.

Protein levels and toxin activity against *C.glabrata* (S-388) were also monitored during downstream processing of the culture. The results in Table 4.6 show that 44% of the protein present at the end of fermentation was lost during filtration through a 0.45 μm cellulose acetate membrane. Concurrent with this result, there was a 20% loss in toxin activity to $8.0x10^4$ units. Of the 168 mg of protein present in the microfiltrate, 102 mg (60%) was detected in the permeate and 45 mg (27%) in the retentate following ultrafiltration. 65% of the toxin activity was detected in the permeate sample ($5.2x10^4$ units) and only approximately 1% in the retentate fraction (1000 units).

Table 4.6 - Protein content during each stage of the downstream processing of *W.mrakii* (K-500) killer toxin produced in the second large-scale fermentation.

Sample	Protein (µg/ml)	Sample Volume (ml)	Total Protein (mg)	Total Units of Activity [a]
Cell-Free Supernatant	7.4	40.0	296	1.0×10^5
Microfiltrate	4.2	40.0	168	8.0×10^4
Ultrafiltrate (Permeate)	2.8	36.5	102	5.2×10^4
Ultrafiltrate (Retentate)	12.8	3.5	45	1.0×10^3

a - total units of activity were calculated on the basis that one unit of activity was equivalent to the volume of toxin required to produce 100% reduction in growth of the sensitive strain *C.glabrata* (S-388). Each solution was concentrated 10-fold prior to assay.

4.7 Discussion

The experiments of Bevan and Makower (1963) showed that the killer toxin was a diffusable factor, secreted into the medium. Production of killer toxins is strongly affected by culture conditions (Woods and Bevan, 1968; Middelbeek *et al.*, 1979), therefore, optimal conditions for each killer yeast studied have to be found empirically.

The production of toxin by the killer yeast *W.mrakii* (K-500) in complex (YEPD), synthetic (SLM) and minimal (YNBGS) media was compared. In each medium, accumulation of the killer factor followed cell growth (Figures 4.1 to 4.3). The measured killing activity increased with cell titre during the exponential phase of growth and levelled off as stationary phase was reached. Detectable levels of toxin activity were apparent at time zero because a 24 hour starter culture was used to initiate growth. It is likely, therefore, that a proportion of toxin was transfered within the inoculum. During the first six hours of growth there was no significant increase in the killing activity, however, as cells progressed through exponential phase and into early stationary phase toxin activity increased, resulting in larger zones of inhibition. It was perhaps surprising that the richest medium, YEPD, did not support the best cell growth (mean generation time of 4.9 hours) yet appeared to induce the greatest killing activity against the indicator strain. Cultures supplemented with yeast extract and peptone have previously been reported to enhance toxin production (Woods and Bevan, 1968; Palfree and Bussey, 1979) which suggested that either, or both, of these components stimulated toxin production or stabilised the killer factor produced. SLM supported growth of the killer yeast (mean generation time of 3.1 hours), however, reduced the activity of the killer factor secreted into the extracellular medium. *W.mrakii* has simple nutritional requirements (Barnett *et al.*, 1983), so good cell growth (mean generation time of 2.6 hours) in the chemically-defined YNBGS was not unexpected. The presence of magnesium sulphate in the medium was thought to potentiate toxin production (O'Leary, 1987), although this effect may be secondary to improved cell growth and biomass accumulation. Its addition to growth media for the production of killer toxins has been reported by other groups (Palfree and Bussey, 1979; Ohta *et al.*, 1984). An improvement in ammonium assimilation can stimulate protein synthesis by the promotion of glutamic acid availibility as a basic intermediate of amino acid precursors

(Grafe, 1982). Therefore, ammonium sulphate was also added to the medium as an additional nitrogen source. Sugisaki *et al.* (1984) had previously reported that its addition induced a five-fold increase in toxin yield and Ouchi *et al.* (1978) that it stabilised the toxin produced.

Use of a minimal medium for toxin production was preferred because potentially it would yield a very high initial specific activity and would allow an integration of the fermentation with downstream processing. A reduction in the amount of extraneous polypeptides and proteins present in the extracellular medium would be beneficial to any subsequent purification steps and throughput on ultrafiltration membranes. The minimal medium YNBGS, was selected as the growth medium for further studies of the killer yeast *W.mrakii* despite the reduced toxin activity detected in comparison to cell-free extracts from cultures in YEPD.

The results in Figure 4.4 further emphasised that the killer factor produced by *W.mrakii* was a growth related response. From a loop-inoculum cell growth was initially very slow and progression of cells into the exponential phase of growth did not occur for approximately 24 hours. The mean generation time of 13.4 hours also reflected the slow growth rate observed. Toxin was produced as cells entered mid to late exponential phase, indicated by the increased growth reduction in a sensitive strain. The data in Figure 4.4 suggested that toxin levels continued to increase as the cells entered stationary phase, however, no firm conclusions could be made because of the low number of samples taken in the latter stages of the fermentation. Levels of protein in the extracellular media reflected both cell growth and toxin production (Figure 4.5) but only very small amounts (1.2 µg/ml) were detectable after 186 hours. It would appear that under these conditions active killer toxin was produced, but because of the poor starting inoculum residual levels of the toxin remained very low. Initiation of future fermentations were made with starter cultures so that biomass accumulation was improved for greater toxin production.

Initial processing of small-scale static cultures of *W.mrakii* involved several stages. Centrifugation was an important first step to remove the bulk of the cellular material produced. Under static conditions the killer yeast grew with a surface pellicle and centrifugation alone failed to remove all viable cells, therefore, a fine filtration step

through 0.45 μm cellulose acetate membranes was necessary. Some loss of activity occurred but at this stage the extent of the loss was not fully appreciated (see section 4.5). The cell-free supernatant was then concentrated five-fold using Amicon ultrafiltration. Preliminary findings (see section 5.5b) suggested that the toxin of *W.mrakii* (K-500) had a molecular weight of approximately 16 kDa and that of another strain of *W.mrakii* (LKB 169), a molecular weight of 11 kDa (Yamamoto *et al.*, 1986a). Therefore, a PM10 membrane was used for ultrafiltration, which had a molecular weight cut-off of 10 kDa. The retentate, material larger than 10 kDa, was further concentrated by freeze-drying and reconstitution of the lyophilisate in a reduced volume of distilled water. Toxin activity was assessed at each stage against the indicator strain *C.glabrata*, using an agar diffusion bioassay (Table 4.2). The results demonstrated successive increases in killing activity in the cell-free supernatant, retentate and in the lyophilisate. Essentially no activity was detected in the permeate, material smaller than 10 kDa, although there were signs of stained (dead) cells around the wells in the assay. This presented us with further evidence that the active toxin molecule was larger than 10 kDa and this simple scheme could be used routinely to produce a partial purification of the killer factor of *W.mrakii*.

To facilitate the production of substantial titres of active killer factor under static conditions, it was necessary to grow the yeast over extended periods of time. In an attempt to reduce fermentation times, a study was conducted to investigate the effect of agitation and oxygenation on toxin activity.

The amount of dissolved oxygen available to the yeast during growth affected the accumulation of the toxin produced by *W.mrakii*. The yeast was grown in three fermenters; fermenter A had '0%' dissolved oxygen present at inoculation (essentially anaerobic), fermenter B was continually aerated and fermenter C was gently agitated for 2 hours prior to inoculation. After addition of the starter cultures, fermenter A was maintained under static conditions, whilst fermenters B and C continued to be gently stirred. The latter was sufficient to prevent pellicle formation on the surface of the broth. Three different patterns of growth were produced, as shown in Figures 4.6 to 4.8. In fermenter C (Figure 4.8), typical growth was produced with cells entering exponential phase after 4 hours and stationary phase at approximately 24 hours. The mean

generation time of cells during active growth was 4.3 hours. Growth of the killer yeast in an essentially anaerobic environment was possible, as demonstrated in fermenter A (Figure 4.6). However, there was a reduction in the maximum cell growth achieved and an increase in the mean generation time to 10.4 hours. The growth of *W.mrakii* with direct aeration, in fermenter B (Figure 4.7) was comparable to that observed in fermenter C. Cell division occurred at a mean generation time of 3.9 hours during exponential growth, however, there was a reduction in the final cell numbers as stationary phase was reached. Assessment of toxin activity during fermentation further exemplified the pattern of killer toxin production as cells progressed through their growth cycle. Measurable levels of the killer factor were observed in each fermentation at approximately the same point, as cells entered their stationary phase of growth. It was not possible, however, to correlate the detected toxin activities with an exact amount of toxin produced by assay of a single volume of cell-free supernatant. If a critical number of toxin molecules were required for inhibition of the sensitive strain, it was possible that at this volume a 'saturation point' had been reached where excess toxin molecules were present (see section 6.6). Therefore, if reduced volumes of supernatants were assayed it would give an indication of any increase in the amount of active toxin present in the extracellular media. By calculation, an estimate of the total units of toxin activity in each fermenter could be made and this was compared to the total amount of protein present (Figures 4.9 to 4.11). A clear relationship was demonstrated between toxin production and the accumulation of extracellular proteinaceous material. In fermenter C, cells entered a definite stationary phase following a period of 48 hours, at which point there was no further production of active toxin and very little increase in extraneous protein (Figure 4.11). However, in fermenters A and B the growth cycle of the killer yeast was affected by the extremes of oxygen available to the cells. In fermenter A the lack of dissolved oxygen present in the media at inoculation, led to a prolonged exponential phase, a reduction in cell numbers and a slow progression of cells into stationary phase. This was reflected in the levels of protein and residual toxin activity detected in the cultures (Figure 4.9). The larger amounts of protein produced in fermenter A suggested that not all protein present could be attributed to active toxin. A response of the cells to the experimental conditions may have led to the synthesis of 'stress proteins'. A variety of stressful treatments, including anoxia, nitrogen starvation,

exposure to ethanol, high temperatures, and the presence of chemical inhibitors, all induce the synthesis of heat-shock proteins in a wide range of organisms (Craig, 1985; Walker and McWilliams, 1989). The anaerobic conditions could, therefore, have induced the production and secretion of additional proteins from the killer yeast. The maximum toxin activity achieved in fermenter B, however, was only 50% of that seen in fermenters A and C, and as fermentation progressed the activity fell to minimum levels after 72 hours (Figure 4.10). These observations can not be explained by the reduction in cell biomass alone, but suggest that the killer factor produced by *W.mrakii* became unstable in the presence of high levels of oxygen and toxin activity was lost. Oxidation of amino acids present in the primary structure of the protein may lead to abberations in the folding and terminal structure of the toxin molecule. It is also possible that the culture conditions led to an increase in the production of extracellular proteases which resulted in the proteolytic breakdown of toxin molecules. The loss of toxin activity observed in the aerated culture was correlated with a reduction in protein in the extracellular medium.

Measurement of the pH throughout the course of a fermentation of the killer yeast *W.mrakii* showed a drop in pH from approximately 5.5 to 2.7 (data not shown). It was possible, therefore, that the killing activity displayed in agar diffusion and microtitre assays, was due to a pH effect rather than the toxin acting directly on the sensitive yeast cell or affecting a metabolic pathway. A simple comparison was made of toxins from killers and non-killers in an attempt to discover if growth inhibition was simply due to acidification of the assay medium. Fermentation supernatant from the non-killer *P.subpellicosa* (NCYC 436) showed a similar pH to that of *W.mrakii* (K-500) yet, did not produce the same degree of killing in the indicator strain *C.glabrata* (S-388). *W.mrakii* produced 93% reduction in growth of the sensitive and *P.subpellicosa* 3% reduction (data not shown). This suggested that only *W.mrakii* actively killed the sensitive strain.

The results of this study demonstrated that the killer yeast *W.mrakii* was 'microaerophilic'. Gentle agitation of the culture produced sufficient oxygen transfer for the growth of the yeast and the production of high levels of toxin in the extracellular

media within 24 to 64 hours of fermentation. Using this information, attempts were made to increase production of active material for future purification work. *W.mrakii* was grown in two one litre fermenters over a three day period and the cultures were combined and processed as described in section 4.5. Table 4.2 documents the residual toxin activity and protein content at each stage of the processing.

Relatively high toxin titres (approximately 13,000 units) were present in both starter cultures, $S1_A$ and $S1_B$, however, the activity was associated with low levels of protein, 1.30 µg/ml and 1.80 µg/ml respectively. Although the combined cultures had a high specific activity of 22000 units/mg of protein, prolonged fermentation may have produced further increases in toxin titres. Following microfiltration a significant loss (25%) in toxin activity was observed and this was associated with a 66% loss in protein. In all likelihood toxin protein was bound to the cellulose acetate membrane which led to the reduced killing activity of the preparation. Woods and Bevan (1968) reported a loss of activity in the *S.cerevisiae* killer toxin on filter sterilisation and suggested that it was due to surface inactivation. Shimizu *et al.* (1985) also reported the binding of killer factors from wine yeasts to nitrocellulose membranes. The cell-free supernatant was concentrated by Millipore ultrafiltration through Pelicon PTGC cassettes. Assay of the permeate, the unconcentrated fraction, suggested that there was no toxin activity present. A microtitre assay revealed that no lethality was produced in a sensitive strain of *C.glabrata.* Protein levels in this fraction were also negligible. However, only 20% of the toxin activity present before ultrafiltration was detected in the retentate. The large losses of protein associated with this step of the purification again suggested adsorption of toxin protein to the cassettes. In hindsight, it was also possible that toxin activity was present in the unconcentrated permeate sample but remained undetected because it fell below the 'threshold limit' of the microtitre assay (see section 6.6). The resultant lyophilisate was reconstituted in 10 ml of distilled water, overall a 120-fold concentration of the killer factor, and was assayed for activity. Although a small volume (6 µl) produced 100% reduction in growth of the sensitive strain, it again appeared that a considerable loss (42%) in activity had occurred during freeze-drying. However, only a small reduction in protein levels was observed. A 'glassy' residue remained following lyophilisation rather than the light, fluffy powder which should have been obtained. This was indicative of the solution thawing out during the freeze-drying process and may

account for some of the losses seen (Harris, 1989). Several reports have also suggested that concentration of killer factors greater than 10-fold (Middelbeek *et al.*, 1979) and 50-fold (Sawant *et al.*, 1989) leads to substantial losses in killer activity. These losses may be explained by the aggregation of toxin molecules which results in the blockage of the receptor recognition sites on the surface of the sensitive yeast strain (Sawant *et al.*, 1989).

Although losses of toxin activity were observed when the downstream processing was more critically appraised, activity remained detectable in the lyophilisate. It was deemed important, therefore, at this stage to produce sufficiently large amounts of this active material for future work. It was proposed to scale-up the fermentation of the killer yeast *W.mrakii* from several smaller fermentations (two to three litres) to 40 litres with the aid of the Fermentation Division at Glaxo Group Research.

The procedure followed during the initial 40 litre fermentation is outlined in Figure 4.13. The running conditions were based on the results of the previous research. A static culture of the killer yeast (250 ml) was grown simultaneously to monitor any differences in cell growth and toxin production. After 24 hours, on-line measurements (absorbance at 600 nm) indicated poor growth of the yeast (an absorbance of 0.1 compared to 0.5 in static culture). It was assumed that if cell growth was reduced then so too would toxin production. The low cell biomass observed was attributed to the very low levels of oxygen available within the fermenter. Although earlier work had suggested that gentle agitation of the culture was sufficient for growth of the killer yeast, such a large vessel required minimal aeration. The fermentation was prolonged for a further 24 hours with aeration of 1.5 litres/min. At completion the cell count of 3.7×10^7 cells/ml was 40% of that achieved in static culture. The fermentation broth was harvested as outlined in section 2.8.2 by centrifugation, microfiltration and ultrafiltration to produce three litres of concentrated material with a molecular weight greater than 10 kDa. The retentate was freeze-dried to produce 59.87 g of lyophilisate. Results in Table 4.3 suggested that very little killer factor was produced by the yeast during fermentation. Unconcentrated samples taken after 48 hours, microfiltration and from the permeate, showed no killing activity against C316. When concentrated only 28% reduction in growth of the indicator strain was observed after 24 hours, however, this did increase to 80% after 48 hours.

The enhanced killing activity *i.e.* more active toxin molecules present in the extracellular medium, was in all likelihood a response to the increase in cell growth between 24 and 48 hours. The low toxin titres produced during the fermentation were further highlighted when compared to the static culture. The same toxic action against C316 was observed in the 24 (100 µl) and 48 hour sample (50 µl). Although the retentate was active against the indicator strain the response observed did not reflect the large concentration step involved. Analysis of the freeze-dried material suggested that a large proportion (77%) of the end-product was carbohydrate. The reduced cell growth meant a high proportion of the media carbohydrate remained unused and was concentrated during processing. Only a small amount of the lyophilisate was proteinaceous (0.06%) which was also indicative of low toxin production.

A second fermentation was performed and the same procedure described above was followed except the culture was aerated at 3.5 litres/min in an attempt to enhance growth of the killer yeast. Cells entered stationary phase at approximately 28 hours and a mean generation time of 4.07 hours was comparable to earlier fermentations. After 48 hours a maximum cell growth of 1.25×10^8 cells/ml was produced. The levels of protein detected in the extracellular medium were constant until late exponential phase when a dramatic increase from 0.8 to 4.8 µg/ml was observed. As cells progressed into stationary phase, there was a further increase to 5.8 µg/ml before levels began to fall (Figure 4.15). Previous studies suggested that the increases in extracellular protein during fermentation of the yeast *W.mrakii* was indicative of toxin production. The culture was processed to produce 25.06 g of freeze-dried material, and at each stage an assessment was made of toxin activity and protein content. Table 4.6 again highlighted problems associated with the processing scheme used. Microfiltration accounted for a 43% loss in protein and a subsequent 20% loss in toxin activity. The adsorption of the killer toxin to the membranes was the most probable cause of these losses. Ultrafiltration appeared to fractionate the cell-free supernatant without significant loss of protein. However, it was surprising to note that the majority of the toxin activity was detected in the permeate fraction (65%), material less than 10 kDa in size. Only 1% of the total activity was found in the retentate. The presence of toxin activity in both the retentate and permeate samples was not unusual. Ideally all molecules greater than 10

kDa will not pass through a membrane during ultrafiltration, whilst those smaller than 10 kDa will. However, due to the distribution of pore sizes in membranes there is also a distribution in the molecular weight of the molecules able to pass through. Thus, the filtrate will contain a certain percentage of molecules with molecular weights less than the nominal cut-off of the membrane and a similar percentage with a higher molecular weight (Harris, 1989). Table 4.7 summarises results from both large-scale fermentations. It was apparent that very low amounts of protein were associated with the killing activity of the yeast *W.mrakii*, with only milligram amounts being produced from 40 litres of culture. The yield was, however, improved five-fold between the two fermentations yet still accounted for less than 1% of the freeze-dried material produced.

The second fermentation yielded approximately only 50% of the material from the first, 25.06 g compared to 59.87 g. The vast difference between the two can probably be attributed to less media carbohydrate being present at the end of fermentation because of the enhanced cell growth of the killer yeast. Attempts were made to 'clean-up' the toxin preparations using dialysis. The process of dialysis involved placing the toxin solution in a bag of semi-permeable membrane which was submerged in distilled water. Small molecules, such as salts and carbohydrates, passed freely across the membrane whilst larger molecules were retained. Lyophilisate (500 mg/ml) from the initial fermentation was reconstituted and dialysed against distilled water for a period of 24 hours. The starting material, dialysate, dialysing solution and washings from the surface of the tubing were assessed for protein and carbohydrate content and toxin activity. The results in Table 4.5 demonstrated that dialysis had successfully removed almost all the carbohydrate and 70% of the protein from the preparation. The dialysis tubing had a nominal molecular weight cut-off of 12-14 kDa, therefore, only large molecular weight proteins would be retained in the dialysate. The killing activity of each solution was assayed against the indicator strain *C.glabrata* (S-388). The starting material produced 85% inhibition in S-388, however, no growth inhibition was produced by the dialysate, dialysing solution or the washings (data not shown). Dialysis, therefore, resulted in the loss of killer activity from the toxin preparation, which suggested that the molecular weight of the active component was certainly less than 12 kDa. If the active toxin was

Table 4.7 - Summary of the production of protein during large-scale fermentations of the killer yeast *W.mrakii* (K-500). Lyophilisate was reconstituted at a concentration of 500 mg/ml and protein content determined using the Pierce microassay technique.

Fermentation	Amount of Protein in Culture Broth (µg/ml) [a]	Total Yield of Protein (mg)	Percentage of Dry Weight
Fermentation 1	0.096	3.83	0.006
Fermentation 2	0.370	14.83	0.06

a - these values were calculated from the amount of protein present in each lyophilisate at completion of processing

removed during dialysis it was expected that activity would be detected in the dialysing solution, however, no concentration of this solution was made as a confirmation.

4.8 Conclusions

The killer yeast *W.mrakii* (K-500) showed comparable growth in a range of media. The minimal medium, YNBGS was chosen for subsequent studies although it did not support the greatest toxin production. Use of a minimal medium would potentially yield preparations of high specific activities which lacked extraneous polypeptides, a benefit to any subsequent purification attempts. Toxin production was a growth-related response and the killer factor was produced as the cells progressed from late exponential to early stationary phase. Protein levels in the extracellular medium mirrored toxin production. An agitated system with low levels of oxygen present led to the highest titres of killer toxin being produced. Anaerobic or oxygenated systems, whilst supporting growth, inhibited toxin production or destabilised the toxin structure.

Losses in protein and, hence, toxin activity during downstream processing suggested that the killer factor was adsorbed to cellulose acetate membranes and to polysulfone ultrafiltration cassettes. Assumptions that the killer factor was larger than 10 kDa were misleading and permeate samples were not fully analysed. Latter work proceeded to suggest that the toxin molecule was in fact smaller than 10 kDa and loss of toxin activity following dialysis supported the hypothesis of a reduced molecular weight.

Further work is necessary to optimise killer toxin production in the yeast *W.mrakii* (K-500) and several problems associated with downstream processing and scale-up production could be addressed.

CHAPTER FIVE

Characterisation and Purification of *Williopsis mrakii* Killer Toxin

5.1 Introduction

In light of the numbers of killer yeasts which have now been formerly identified, only a few killer toxins have been fully characterised. The most notable is that of the *S.cerevisiae* K1 killer yeast. Palfree and Bussey (1979) isolated a proteinaceous killer toxin from concentrates of the extracellular medium and determined its molecular weight to be 11.5 kDa by SDS-PAGE and amino acid analysis. Its activity was stable over only a narrow temperature and pH range. The killer toxin produced by a strain of *W.mrakii* (LKB 169) was isolated and partially purified by Yamamoto *et al.* (1986a). It was found that the toxin was a molecule of 10.7 kDa which, in contrast to the findings of Palfree and Bussey (1979), was stable over a wide range of temperature and pH.

The aim of this work was to determine the biochemical nature of the killer factor produced by *W.mrakii* (K-500), assess its pH and temperature stability and to attempt a partial purification of the killer toxin using the techniques of FPLC and gel filtration chromatography.

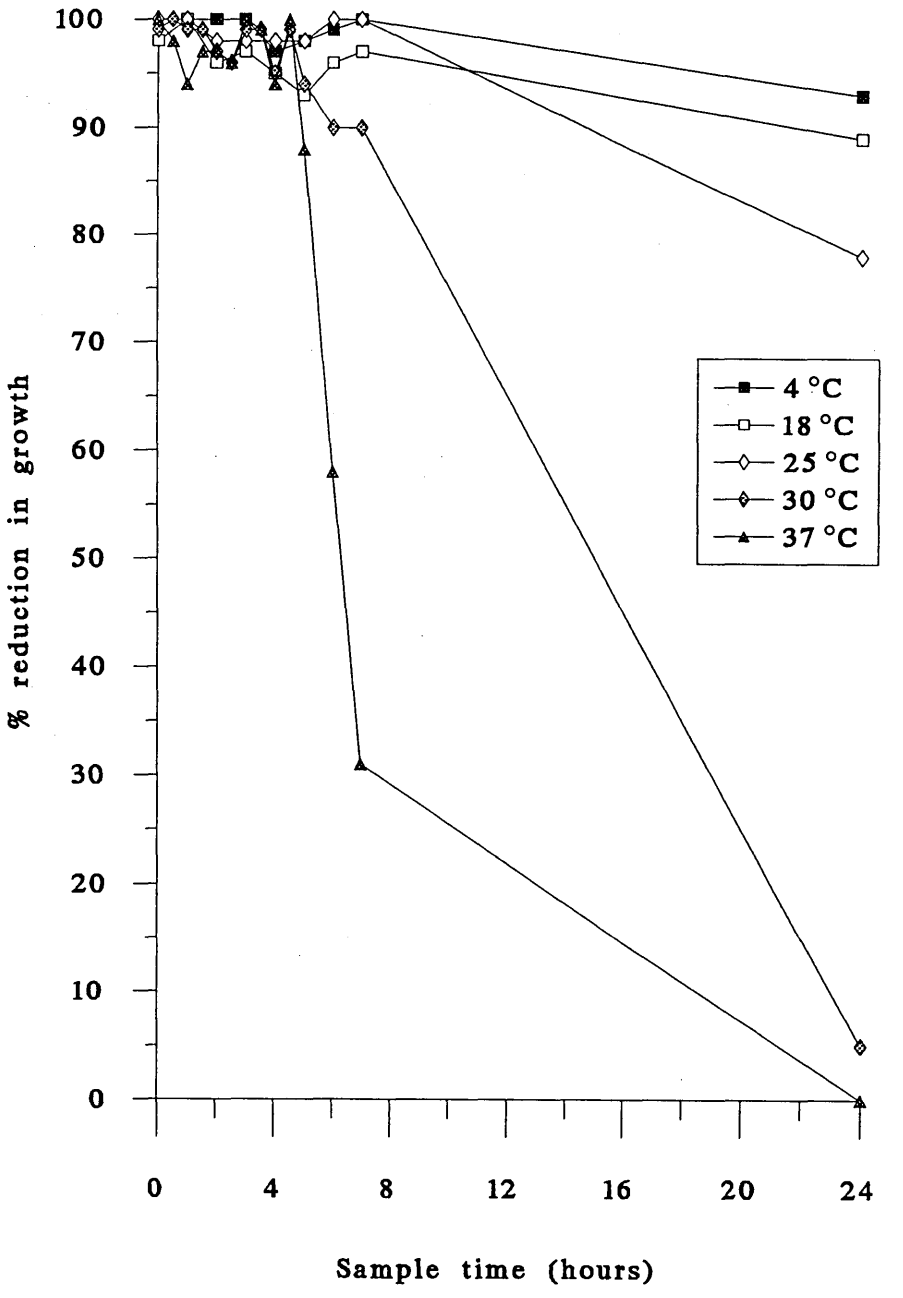
5.2 Temperature Stability of *W.mrakii* (K-500) Killer Toxin

Crude toxin was concentrated 20-fold by freeze-drying cell-free supernatants and reconstituting the resultant lyophilisate in distilled water. Samples of the toxin preparation were incubated at a range of temperatures (section 2.9.1) and at specific intervals, aliquots were removed and assayed for activity using a microtitre plate assay (section 2.6.3).

Figure 5.1 shows the residual activity which remained after incubation of the toxin at 4, 18, 25, 30 and 37^{o}C. The activity was expressed in terms of the percentage reduction in growth produced in a sensitive strain.

The results showed that toxin activity was stable at 4^{o}C and 18^{o}C (ambient temperature) over a 7 hour period and only a 10% reduction in its activity occurred over a 24 hour period. The activity at 25^{o}C was stable for 7 hours, but a 20% loss resulted after 24 hours. At 30^{o}C a rapid loss was seen in residual toxin activity between 7 and 24 hours, with only 5% of the activity remaining after the latter time. At 37^{o}C toxin

Figure 5.1 - Stability of the crude toxin from *W.mrakii* (K-500) to a range of incubation temperatures. At intervals residual toxin activity was measured against a sensitive strain *C.glabrata* (S-388) using a microtitre bioassay.



lethality decreased rapidly between 4 and 7 hours (70% loss) and the toxin was found to be inactive after an incubation of 24 hours at this temperature.
Temperatures of 50°C and 70°C resulted in a complete loss of toxin activity after incubation for 15 minutes, and the toxin was also unstable to boiling (data not shown).

5.3 pH Optimum of *W.mrakii* (K-500) Killing Activity

Crude toxin was prepared from a culture of K-500, which had been incubated with gentle agitation at 25°C for 72 hours. The effect of pH variation on the killing of *C.glabrata* (S-388) and a clinical isolate, *C.albicans* (S-214392), was examined using the agar diffusion bioassay (see Section 2.9.2). The results are shown in Table 5.1.

The killer factor was more active against S-388 than the clinical isolate S-214392, a much larger zone of inhibition, 16.5 mm compared to 11.0 mm, was produced at this pH. However, it can be seen that the toxin activity against both sensitive strains decreased as the pH of the assay medium was increased, and complete loss of activity occurred at pH 5.0. At the higher range of pH values investigated, 7.0-8.0, S-388 failed to grow as a lawn within the agar. The response of both sensitive strains to the action of the killer toxin suggested that a pH of 3.5 was optimal for its killing action.

5.4 pH Stability of *W.mrakii* (K-500) Killer Toxin

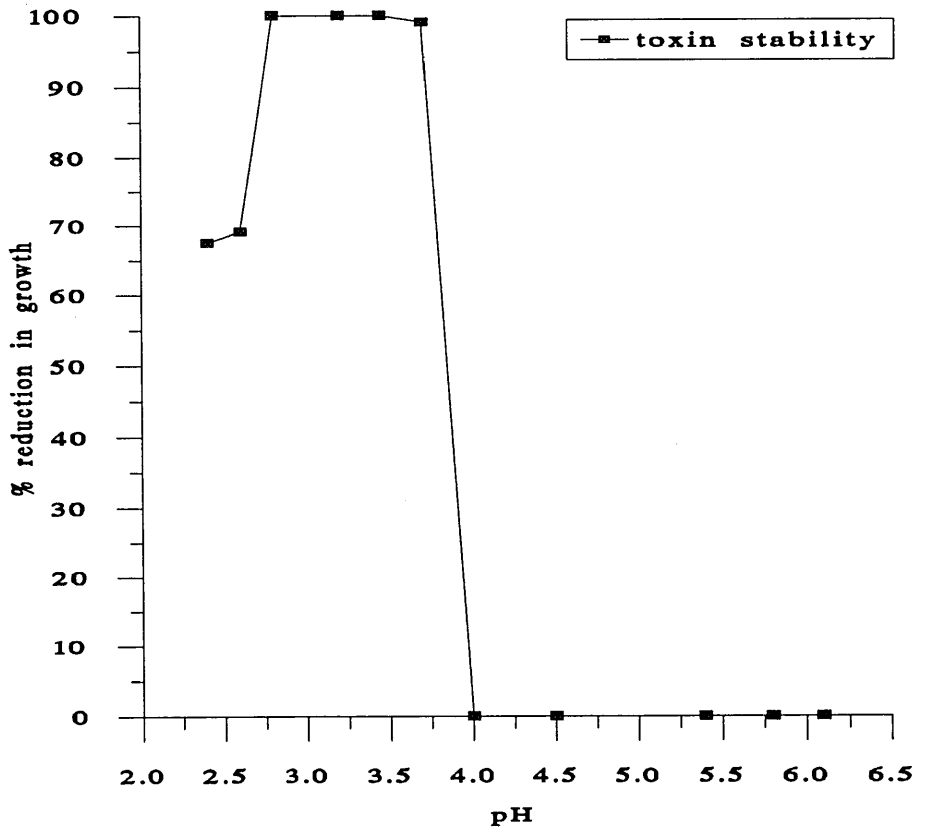
W.mrakii (K-500) killer toxin was buffered over a range of values, pH 2.4 to 6.1, using 0.1 M citrate-phosphate buffer (see section 2.9.3). The solutions were incubated at ambient temperature (18°C) for 18 hours and the remaining toxin activity assessed using a microtitre assay (section 2.6.3). The percentage reduction in growth of the indicator strain, *C.glabrata* (S-388), produced by each solution, was calculated with respect to toxin-free buffer controls of equivalent pH.

Figure 5.2 shows the relative killing activity of each solution when compared to unbuffered toxin (pH 2.3). The killer factor of *W.mrakii* was stable over the pH range 2.4 to 4.0. Between pH 2.8 and 4.0, 100% of the activity was detected, however, at lower values of pH 2.4 to 2.6, only 70% of the original activity was apparent. Inactivation of the killer factor occurred at values greater than pH 4.0.

Table 5.1 - Effect of pH on the killing activity of *W.mrakii* (K-500). The pH of the assay medium was buffered to a range of values and 30µl of crude toxin was added to wells cut in the seeded agar. Subsequent zones of inhibition (mm) were measured after incubation at 25°C for 72 hours.

Sensitive Organism	pH of Media									
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
C.glabrata (S-388)	16.5	15.5	15.0	0	0	0	0	No Growth	No Growth	No Growth
C.albicans (S-214392)	11.0	10.5	9.5	0	0	0	0	0	0	0

Figure 5.2 - The effect of pH on toxin stability. Concentrated toxin was buffered to a range of pH values with 0.1 M citrate-phosphate buffer. After incubation at 18°C for 18 hours, toxin activity was assessed using a microtitre assay.



5.5 PhastSystem Analysis of *W.mrakii* (K-500) Killer Toxin

(a) Isoelectric Focussing (IEF)

IEF is a high resolution technique for separating proteins on the basis of their isoelectric points (pI) and the Pharmacia PhastSystem allowed rapid analysis of toxin preparations. A partially purified sample of the killer toxin (cell-free supernatant which had undergone Amicon ultrafiltration, section 2.8.1) was loaded onto the gradient gel IEF 3-9 (lanes 2 and 5) along with a medium (YNBGS) control (lane 4) and a sample of toxin which had been boiled (lane 3). Calibration markers with known isoelectric points were run alongside (lanes 1 and 8). After focussing the gel was developed using a silver staining technique (see section 2.9.5b).

Plate 5.1 shows the separation of proteins present within the toxin preparation and Table 5.2 the distances moved by the markers. Using a calibration graph (Figure 5.3), an estimate could be made of the isoelectric points of the components of the partially purified toxin (Table 5.3). A strong and diffuse band was seen at a distance of 20.0 mm from the cathode and this coincided with a pI of 6.15, the single band seen in the media control migrated the same distance. A second crisp band was observed at a distance of 31.5 mm (pI 4.35) from the cathode and two weakly stained doublets were apparent at 22.5 mm (pI 5.80) and 23.5 mm (pI 5.65), and 29.0 mm (pI 4.75) and 30.0 mm (pI 4.70) respectively. No bands were visible in the sample of toxin which had previously been boiled.

(b) Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Rapid SDS-PAGE electrophoresis to accurately determine molecular weight distributions was also possible using the Pharmacia PhastSystem. The same partially purified sample of killer toxin was prepared by addition to boiling mix and was heated to $100^{o}C$ for 3 minutes (see Section 2.9.5a). It was noted that the normal colour of the solution, due to the Coomassie Blue in the mix, was in fact changed from blue to yellow on addition of the toxin. The samples and molecular weight markers were loaded onto a homogenous PhastGel and after separation developed by silver staining.

Plate 5.1 - PhastSystem Isoelectric Focussing of *W.mrakii* (K-500) killer toxin.

Lanes **1** and **8** - broad pI calibration markers

Lanes **3** and **6** - partially purified killer toxin

Lane **5** - boiled toxin

Lane **4** - media (YNBGS) control

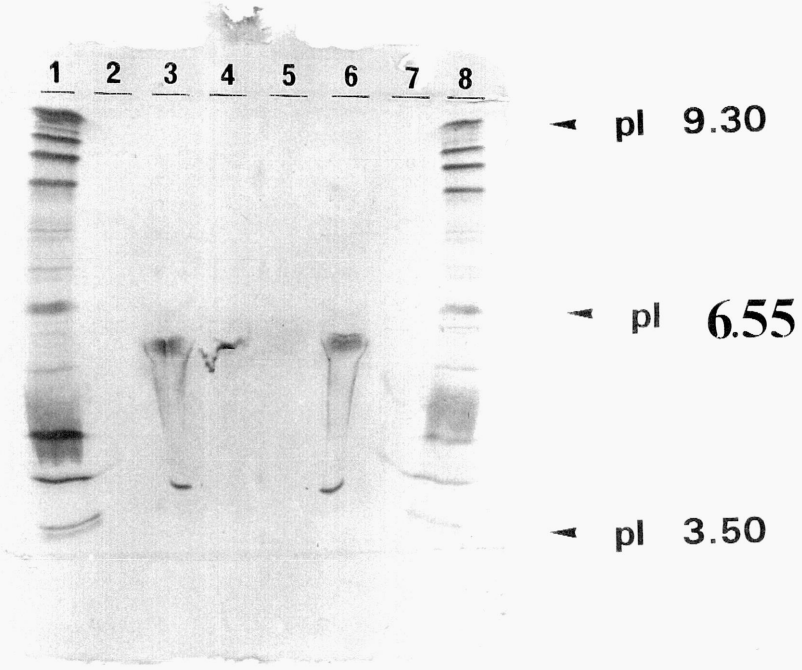


Figure 5.3 - Calibration graph to estimate the isoelectric points of components in a partially purified preparation of *W.mrakii* (K-500) killer toxin.

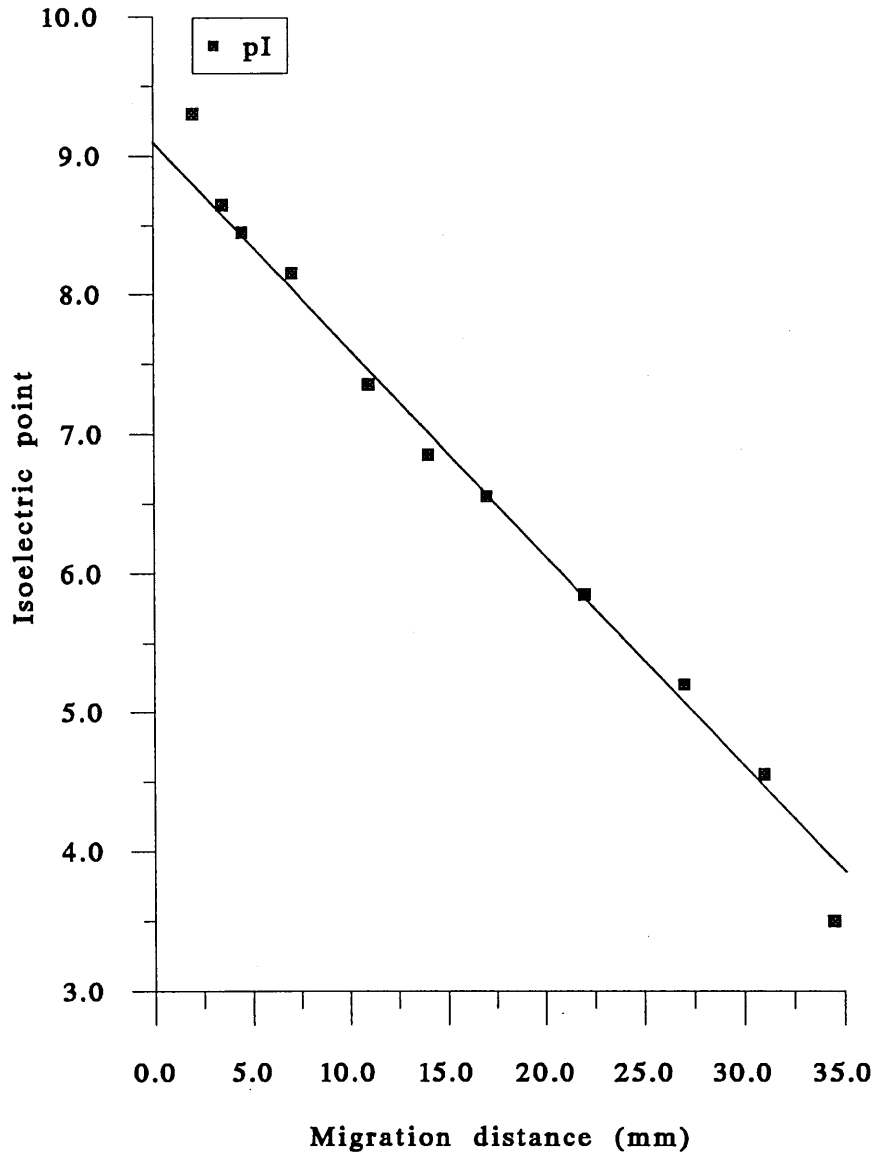


Table 5.2 - Table showing the isoelectric points (pI) and migration (mm) of the molecular weight markers used for calibration of the PhastSystem IEF gel (IEF 3-9).

Name of Marker	pI	Migration Distance (mm)
Amyloglucosidase	3.50	34.5
Soybean Trypsin Inhibitor	4.55	31.0
β-lactoglobulin A	5.20	27.0
Bovine Carbonic Anhydrase B	5.85	22.0
Human Carbonic Anhydrase B	6.55	17.0
Horse Myoglobin (acidic)	6.85	14.0
Horse Myoglobin (basic)	7.35	11.0
Lentil Lectin (acidic)	8.15	7.0
Lentil Lectin (middle)	8.45	4.5
Lentil Lectin (basic)	8.65	3.5
Trypsinogen	9.30	2.0

Table 5.3 - The estimated isoelectric points (pI) of the components of the partially purified *W.mrakii* (K-500) killer toxin (calculated from the calibration graph shown in Figure 5.3).

Migration Distance (mm)	pI	Migration Distance (mm)	pI
20.0	6.15	29.0	4.75
22.5	5.80	30.0	4.70
23.5	5.65	31.5	4.35

Inconsistent results were obtained, however, a very weak band was apparent which coincided with a molecular weight of approximately 16 kDa (data not shown). Subsequent attempts to repeat this separation on PhastGels and Mini-gels were unsuccessful.

5.6 Effect of Proteolytic Enzymes on Toxin Activity in Two Strains of *W.mrakii*

(a) Prior to investigation of the effects of pronase, pepsin and trypsin on toxin activity, the enzymes were standardised against a haemoglobin substrate (section 2.9.4). The concentration of each enzyme which hydrolysed the substrate to the same extent was determined and used in studies to further characterise the killer toxin.

It was found that concentrations of pepsin (0.50 mg/ml) and pronase (2.25 mg/ml) produced the same extent of hydrolysis, which correlated to a measured absorbance of 1.000 at 280 nm (Figure 5.4). Trypsin, however, at the concentration assayed, failed to hydrolyse the haemoglobin to the same extent. Increasing the concentration of the enzyme failed to produce a comparable response, therefore, only the effect of pronase and pepsin on the killer factor was further investigated.

(b) The effects of native and denatured enzymes on killer activity in *W.mrakii* (K-500 and K-LKB) was assessed relative to the appropriate control solutions (section 2.9.4). The residual toxin activity was determined using a microtitre assay (section 2.6.3).

The results in Figure 5.5 suggested that from a single microtitre assay the killer factor in both strains of *W.mrakii* was resistant to the action of the proteolytic enzymes tested. 100% of the toxin activity was maintained after incubation with native and denatured enzymes, or control solutions, distilled water (pepsin control) and 5 mM $CaCl_2$ (pronase control). K-500 killer factor was inactivated by high temperature treatment, however, K-LKB maintained 60% and 80% of its activity following incubation with pepsin and pronase respectively.

Figure 5.4 - Standardisation of the proteolytic enzymes against a haemoglobin substrate. Increasing concentrations of pepsin, pronase and trypsin were incubated with the substrate for 18 hours at 20°C. The absorbance of TCA-soluble protein, at 280 nm, was used to indicate the extent of hydrolysis.

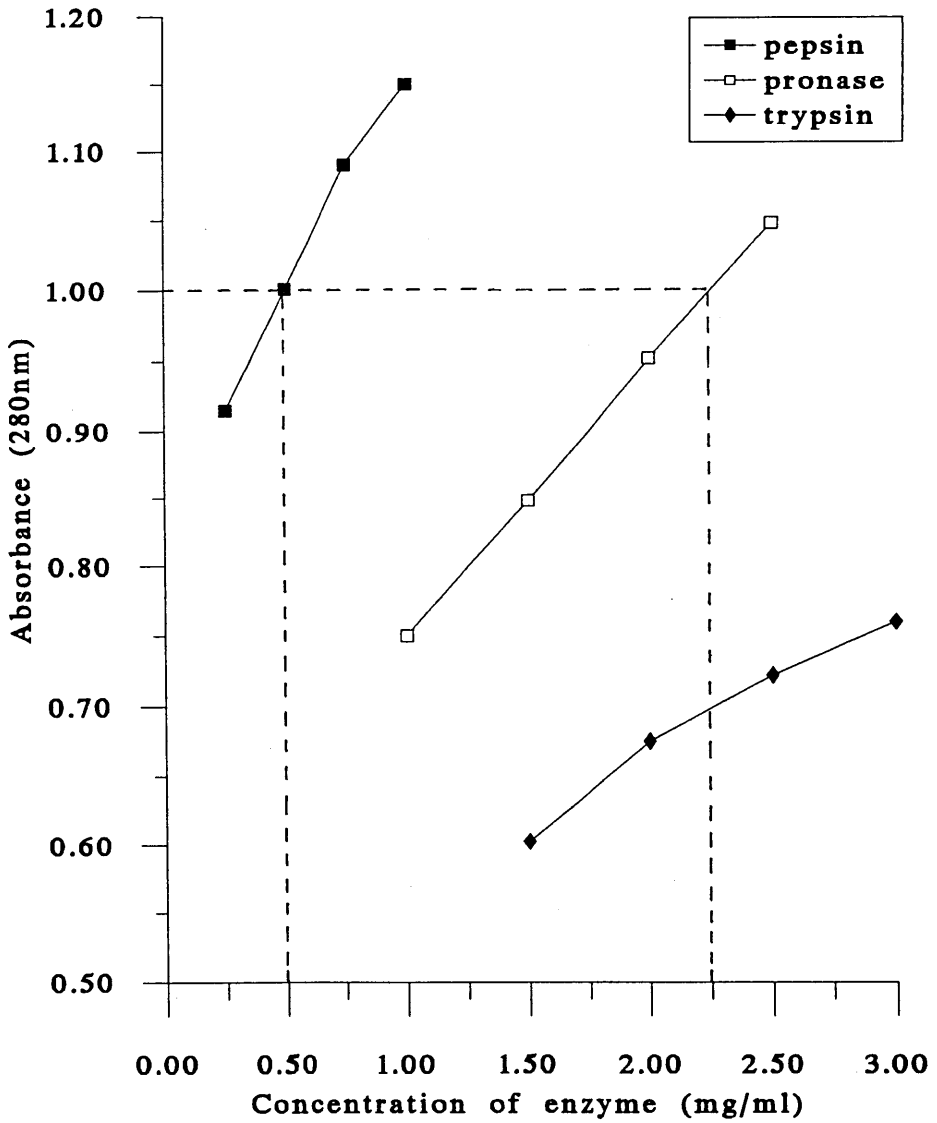
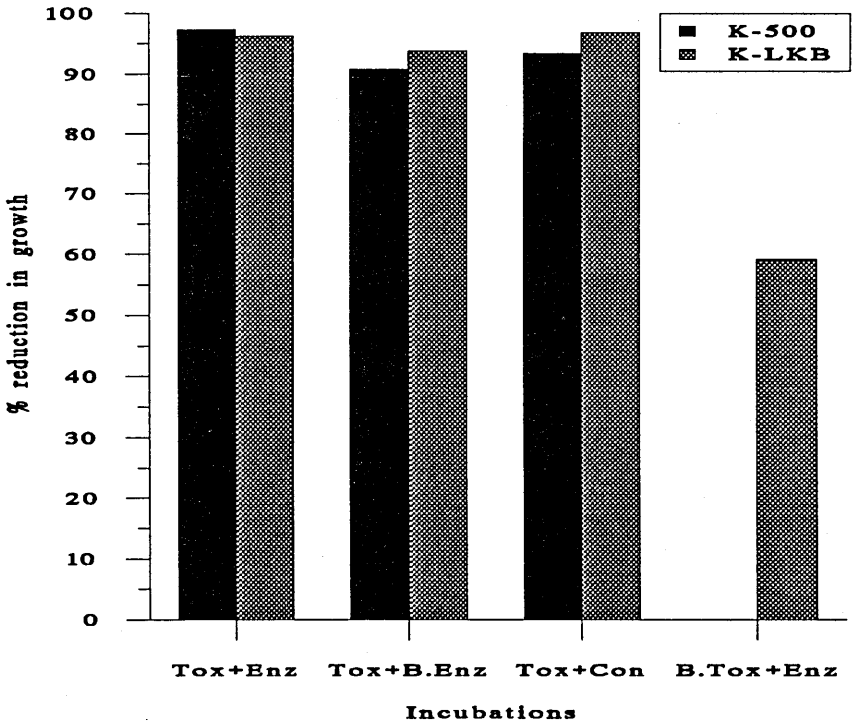


Figure 5.5a - The effect of pepsin (0.50 mg/ml) on the activity of *W.mrakii* K-500 and K-LKB killer toxins. Each solution was incubated at 20°C for 18 hours and the remaining toxin activity was assessed against the sensitive strain *C.glabrata* (S-388) using a microtitre assay.



KEY :

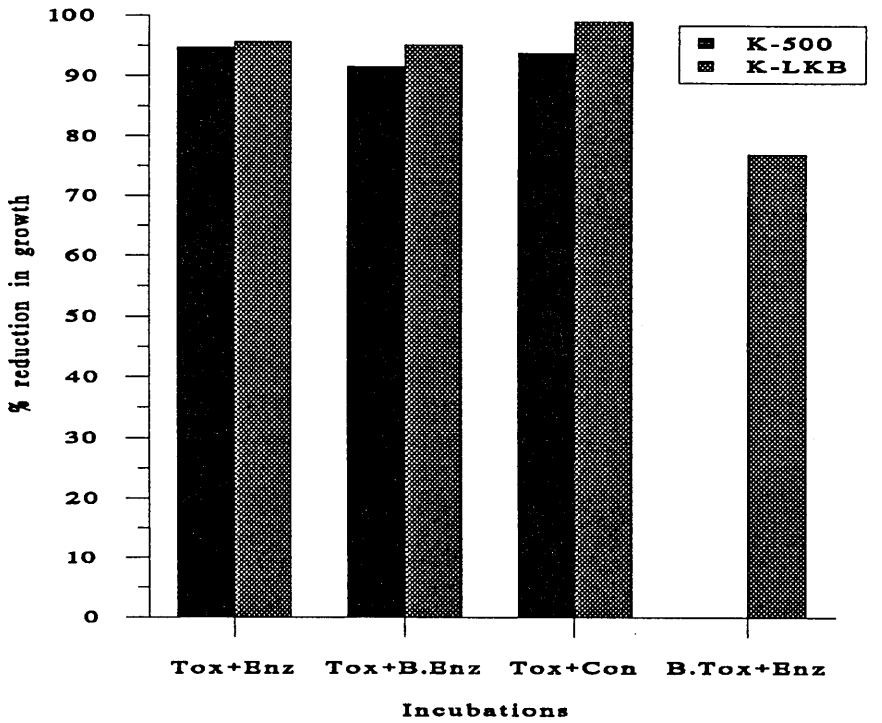
Tox + Enz - killer toxin incubated with pepsin (0.50 mg/ml)

Tox + B.Enz - killer toxin incubated with heat denatured enzyme

Tox + Con - killer toxin incubated with distilled water control

B.Tox + Enz - heat-treated killer toxin incubated with pepsin (0.50 mg/ml)

Figure 5.5b - The effect of pronase (2.25 mg/ml) on the activity of *W.mrakii* K-500 and K-LKB killer toxins. Each solution was incubated at 20°C for 18 hours and the remaining toxin activity was assessed against the sensitive strain *C.glabrata* (S-388) using a microtitre assay.



KEY :

Tox + Enz - killer toxin incubated with pronase (2.25 mg/ml)

Tox + B.Enz - killer toxin incubated with heat denatured enzyme

Tox + Con - killer toxin incubated with 5 mM $CaCl_2$ control

B.Tox + Enz - heat-treated killer toxin incubated with pronase (2.25 mg/ml)

5.7 Fast Protein Liquid Chromatography (FPLC) Purification of *W.mrakii* Killer Toxin

A Mono Q anion-exchange column was used for separation of the components of *W.mrakii* killer toxin. The toxin preparation used in the following separations was cell-free supernatant concentrated by Millipore ultrafiltration (10 kDa cut-off). The retentate was concentrated further by freeze-drying and the lyophilisate reconstituted in a reduced volume of distilled water. The toxin preparations were applied to the column and eluted with a range of buffer systems, see section 2.10.1 for technical details.

Buffer system 1

Buffer A : 20 mM TRIS, pH 8.0

Buffer B : 20 mM TRIS + 1.0 M NaCl, pH 8.0

The first buffer system (pH 8.0) used, produced the elution profile seen in Figure 5.6a and five main protein peaks were eluted from the column. A large peak of unbound material (A) passed straight through the column, followed by a distinct peak (B) which was removed by a 15-20% gradient of Buffer B. A smaller peak (C) was removed by 25% Buffer B and two others, tightly bound to the column, were removed by 100% Buffer B. Fractions from peaks A-D were assayed for activity using a microtitre assay (Figure 5.6b). Increasing 'activity' was displayed by fractions from peak B through to peak D and those from the initial unbound material showed only 40% reduction in growth of the sensitive strain.

Buffer System 2

Buffer A : 20 mM *N*-methylpiperazine, pH 4.0

Buffer B : 20 mM *N*-methylpiperazine + 1M NaCl, pH 4.0

The second separation of toxin proteins used an acidic (pH 4.0) buffer system which produced a similar, but less defined, elution profile (Figure 5.7a) than that obtained from buffer system 1. Five peak areas were eluted (A-E), however fractions were only collected from the peaks A (unbound material) and D (removed by 25% gradient of Buffer B) to assay for activity (Figure 5.7b). Microtitre assays showed that the bulk of the activity was eluted in the unbound fraction, approximately 100% reduction in

Figure 5.6a - Elution profile of *W.mrakii* (K-500) killer toxin on a Mono Q HR 5/5 column using a 20 mM TRIS +/- 1.0 M NaCl, pH 8.0 buffer system.

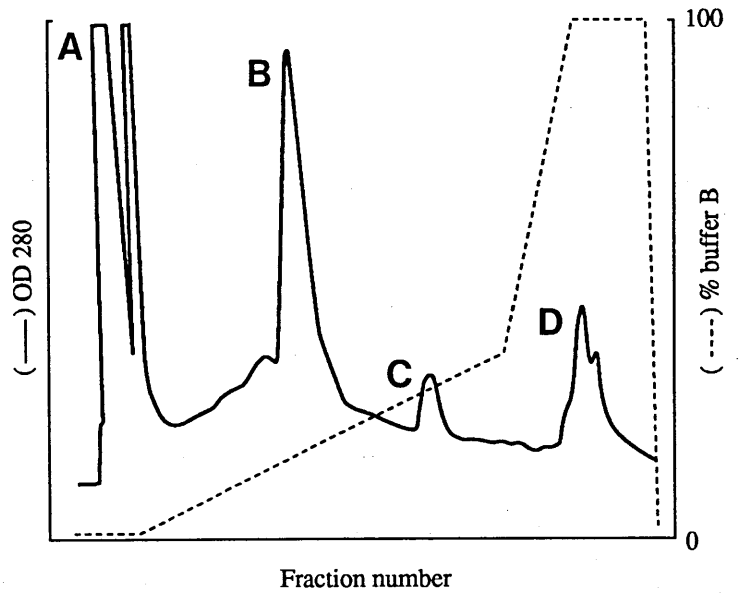


Figure 5.6b - Toxin activity of fractions collected from peaks A-D was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.

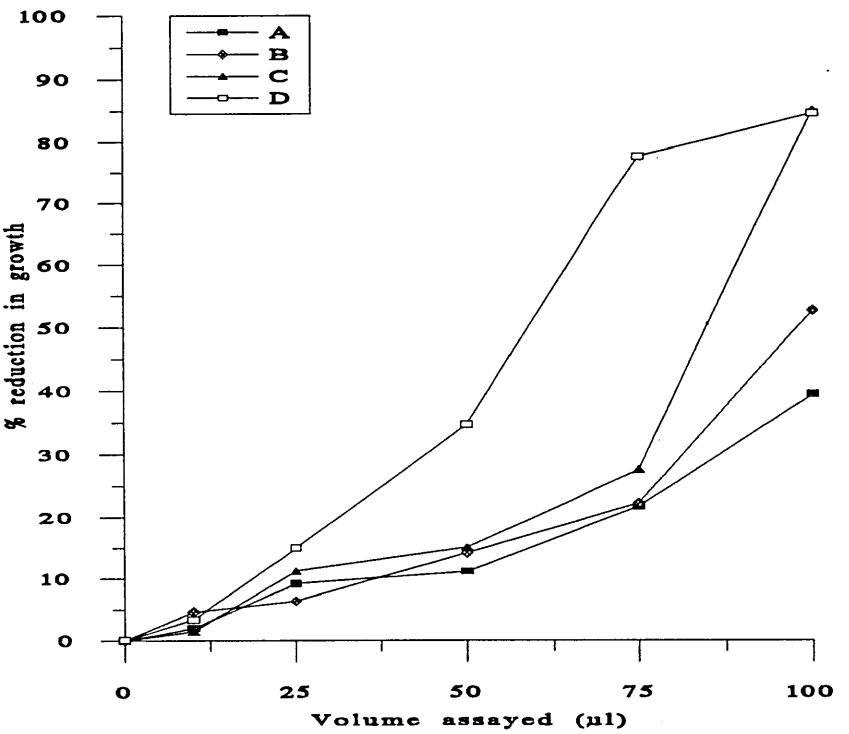


Figure 5.7a - Elution profile of *W.mrakii* (K-500) killer toxin on a Mono Q HR 5/5 column using a 20 mM *N*-methylpiperazine +/- 1.0 M NaCl, pH 4.0 buffer system.

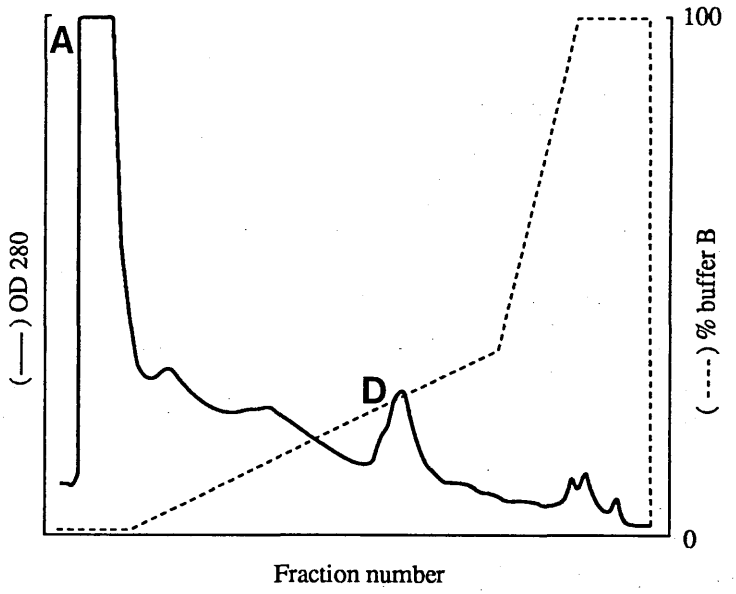
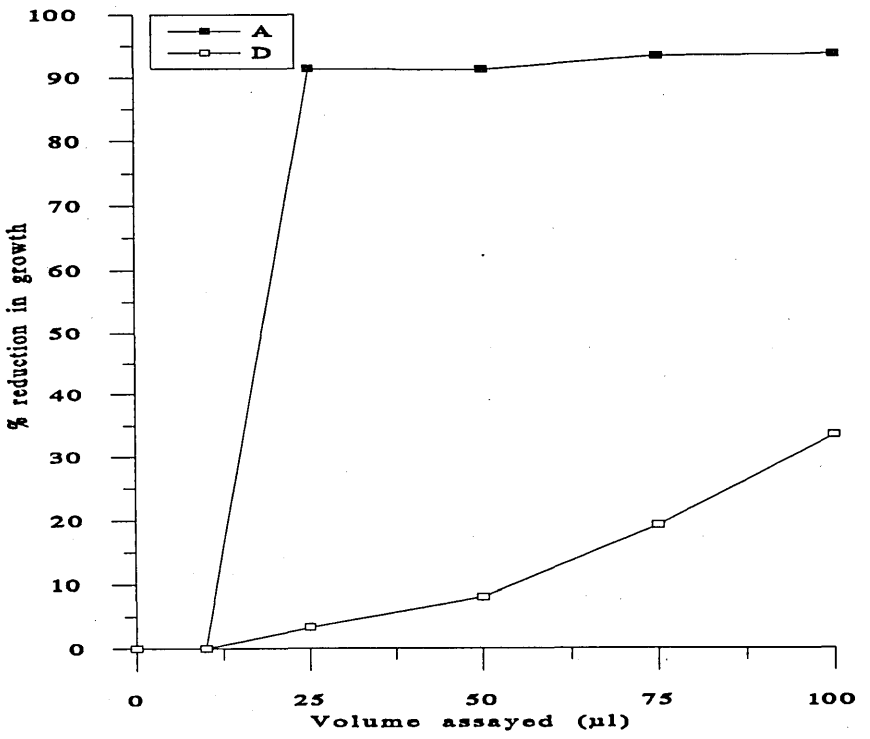


Figure 5.7b - Toxin activity of fractions collected from peaks A and D was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.



growth of the sensitive was produced by 25 µl of this sample. This compared to fractions from peak D in which 100 µl produced a 40% reduction in sensitive growth.

Buffer System 3

Buffer A : 20 mM bis TRIS, pH 6.0

Buffer B : 20 mM bis TRIS + 1.0 M NaCl, pH 6.0

The next separation used an intermediate (pH 6.0) buffer system which produced a similar elution profile (Figure 5.8a) of 5 main peak areas (A-E). Fractions (1 ml) were collected during the elution programme and all were assayed for activity by the microtitre assay (Figure 5.8b). Strong activity was recorded in a single fraction, fraction 3, and moderate activity in fractions 30-39. A repeat of this assay verified that sample 3, a fraction of the unbound material, produced lethality in a sensitive strain. The increasing activity from fraction 20 onwards, especially that observed in fractions 30-39, suggested an effect of increasing salt concentrations in the elution buffer. This was verified by assaying fractions from a blank gradient control against the same indicator strain (data not shown).

Buffer System 4

Buffer A : 20 mM bis TRIS propane, pH 6.5

Buffer B : 20 mM bis TRIS propane + 1.0 M NaCl, pH 6.5

Elution of proteins with a pH 6.5 buffer system (Figure 5.9a) resulted in a profile with 7 main peak areas (A-G). A large unbound sample was removed (A) and 4 other distinct protein peaks (B-E) were eluted by the linear gradient of buffered NaCl. A significant amount of protein was tightly bound to the column (F and G), and was removed with 100% Buffer B. The activity of all the fractions was assayed (Figure 5.9b). No significant toxin activity was observed in any of the fractions, although traces were apparent in the unbound fractions 1 and 2. The same pattern of increased growth inhibition produced by the latter fractions was observed.

Figure 5.8a - Elution profile of *W.mrakii* (K-500) killer toxin on a Mono Q HR 5/5 column using a 20 mM bis TRIS +/- 1.0 M NaCl, pH 6.0 buffer system.

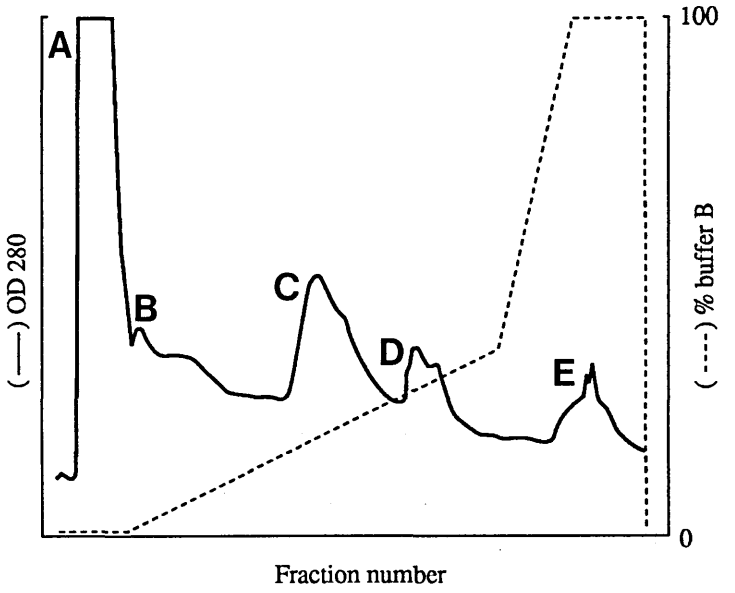


Figure 5.8b - Toxin activity of eluted fractions 1-39 was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.

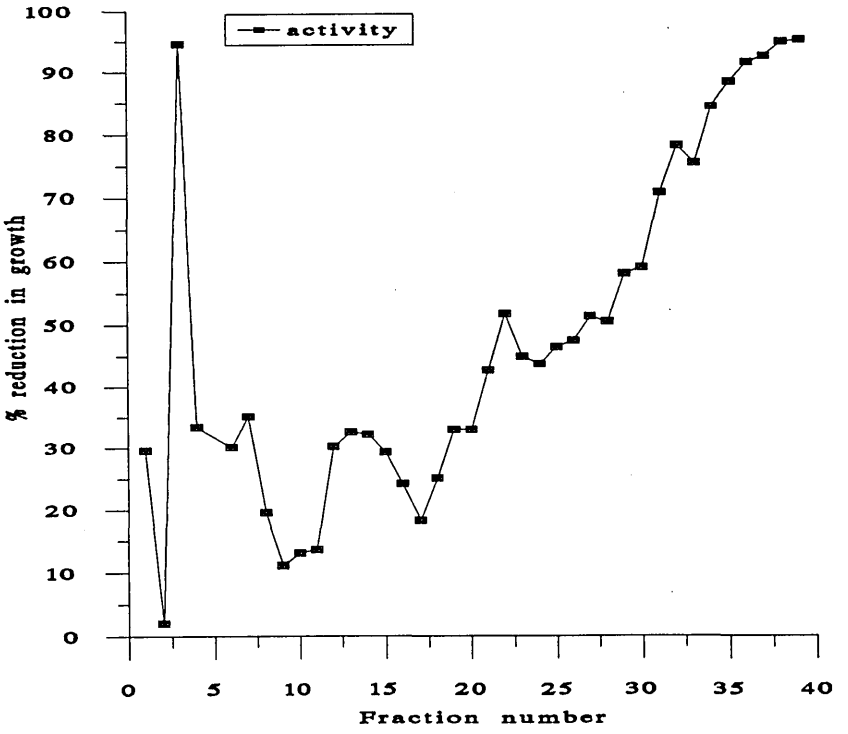


Figure 5.9a - Elution profile of *W.mrakii* (K-500) killer toxin on a Mono Q HR 5/5 column using a 20 mM bis TRIS propane +/- 1.0 M NaCl, pH 6.5 buffer system.

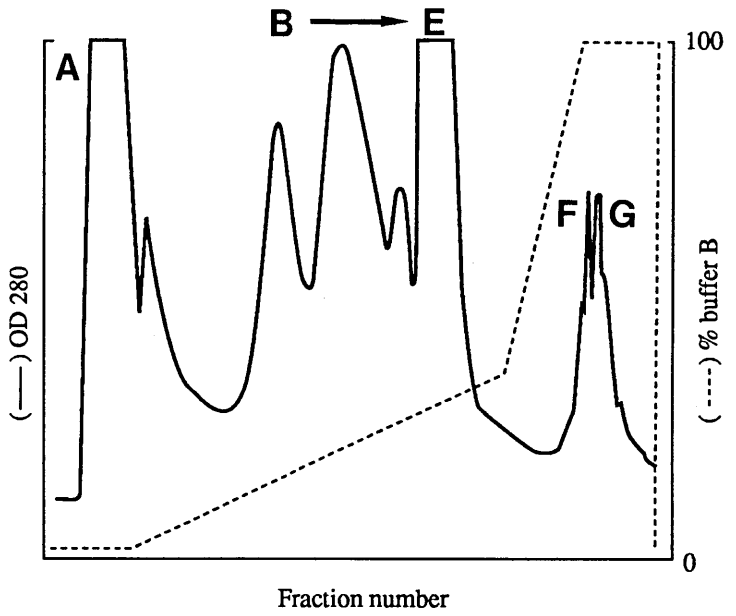
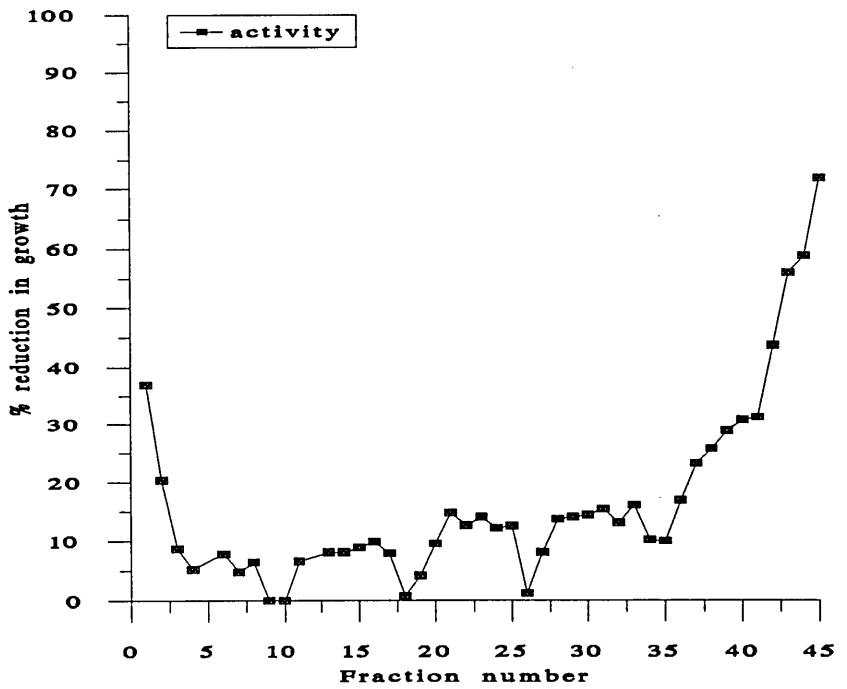


Figure 5.9b - Toxin activity of eluted fractions 1-45 was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.



5.8 Development of a Colourimetric Assay of Toxin Activity

The tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) is a pale yellow substrate which is changed to a dark blue product (MTT-Formazan) through its reduction by the mitochondrial dehydrogenase enzymes of living cells (Slater *et al.*, 1963). The aim was to use this redox indicator to develop a rapid quantitative assay of activity which would detect living, but not dead cells, and accurately reflect sensitive cell viability after toxin treatment.

(a) A comparison was made of the ability of live and dead cells to convert MTT to MTT-Formazan. The relationship between cell numbers and the amount of MTT-Formazan generated was also investigated. Increasing numbers (approximately $5.0x10^6$-$5.0x10^7$) of live and heat-killed *Candida* blastospores were incubated with toxin-free media prior to incubation with the MTT dye (section 2.6.4). The absorbance (570 nm) of the resultant supernatants was measured, which correlated to the extent of MTT conversion to MTT-Formazan.

From Figure 5.10 it can be seen that the absorbance produced was proportional to the numbers of live cells present in the assay, however, there was no significant conversion of the dye by cell numbers below $1.0x10^7$. The results also demonstrated that dead cells produced a negligible response.

(b) Sensitive cells of *C.glabrata* (S-388) were incubated with different amounts of a concentrated crude toxin preparation (freeze-dried cell-free supernatant) of *W.mrakii* (K-500), and the effect on the reduction of the MTT dye was assayed as outlined in section 2.6.4. The absorbance (570 nm) of each incubation was measured and the results recorded in Figure 5.11.

Results showed that the assay was sensitive to the presence of toxin from the killer yeast strain K-500 and subsequently the conversion of MTT to MTT-Formazan was greatly affected. As the amount of toxin in the assay system was increased then less MTT-Formazan was produced, thus, the measured absorbance decreased.

(c) It was necessary to correlate the reduction in conversion of the dye produced by the killer factor, with a loss of mitochondrial enzyme activity and, hence, sensitive cell

Figure 5.10 - Relationship between the number of live cells and MTT-Formazan production. Log-phase cells of *C.glabrata* (S-388) were incubated with MTT for 2 hours at 30^{o}C and reduction to MTT-Formazan was measured spectrophotometrically at 570 nm. A comparison was made of heat killed (70^{o}C for 20 minutes) blastospores.

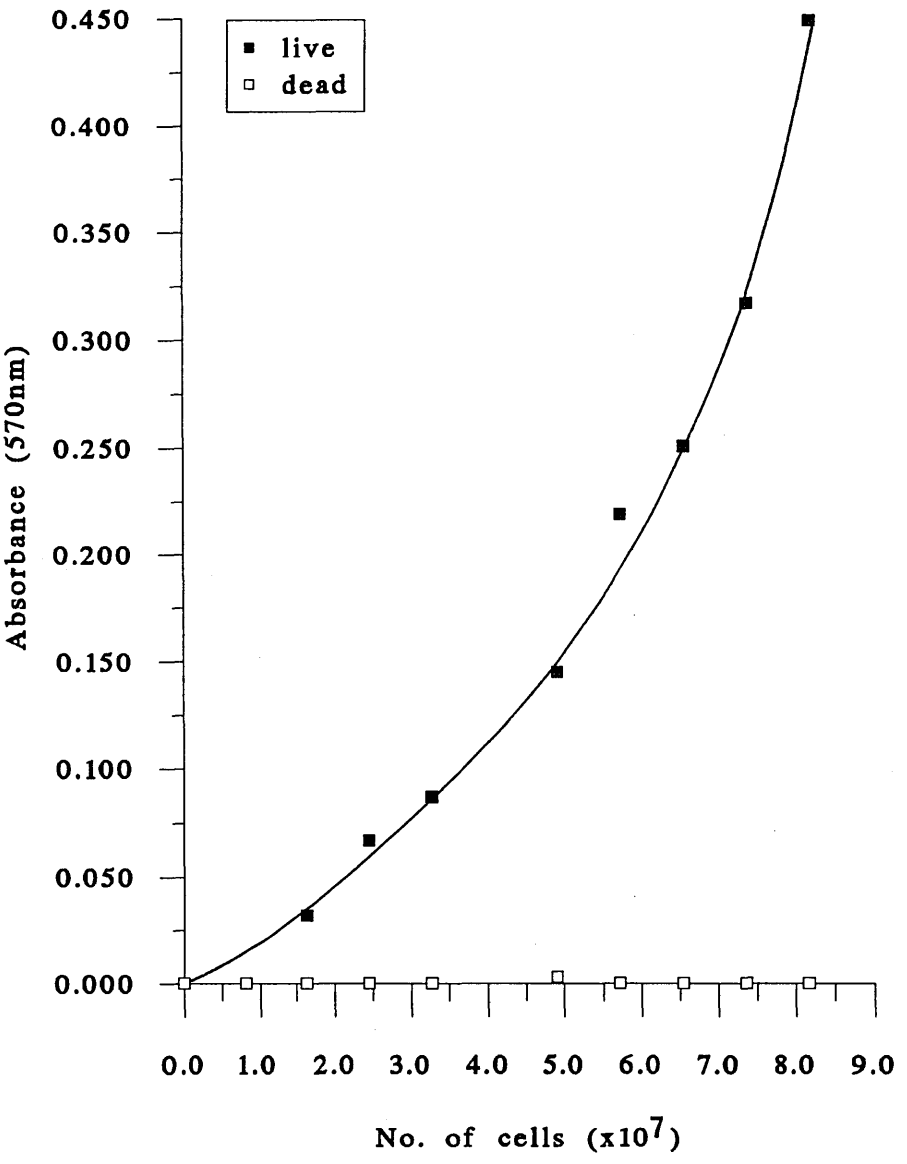
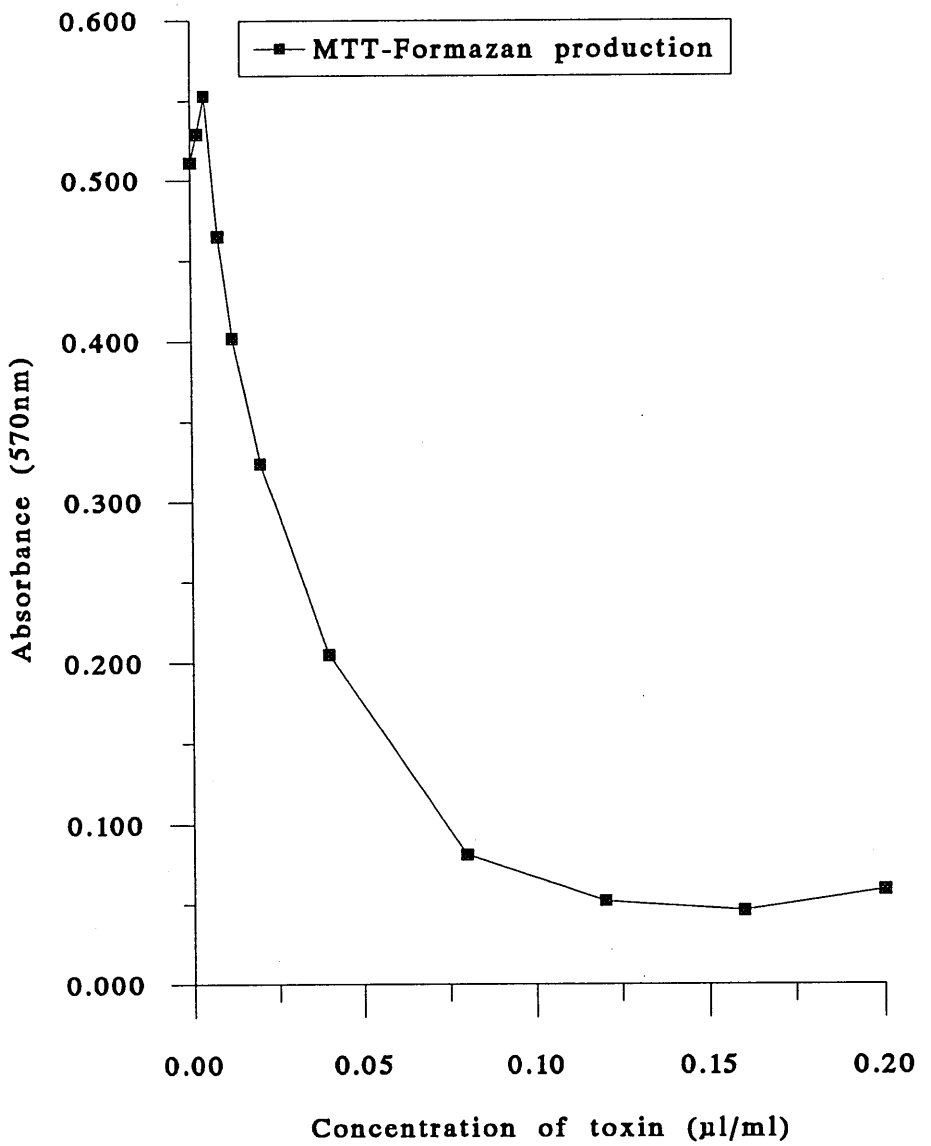


Figure 5.11 - Sensitvity of *C.glabrata* (S-388) blastospores to *W.mrakii* (K-500) killer toxin. Log-phase cells ($1.1x10^8$ cells/ml) of S-388 were exposed to different concentrations of killer toxin for 90 minutes at 20^oC. After incubation, MTT-Formazan production was measured spectrophotometrically at 570 nm.



viability. A subsequent experiment attempted to link the reduction in MTT-Formazan production in toxin-treated cells with the number of viable cells remaining, by plating out dilutions from each incubation on SDA.

Figure 5.12 shows the percentage reduction in viability produced by K-500 killer toxin as measured by the colourimetric MTT assay and a more traditional method which measured colony forming activity on agar plates. The effect on viability was measured with respect to a toxin-free control in each case. A good correlation between the two methods was displayed and both were sensitive to the increases in toxin concentration. The numbers of viable cells decreased, as did the measured absorbance of the final supernatants, as the amount of toxin present in the assay increased. This indicated a direct relationship between sensitive cell viability and the conversion of MTT to MTT-Formazan.

(d) A time-course was also conducted to investigate the relationship between the period of toxin exposure and sensitive cell viability. The sensitive cells of *C.glabrata* were incubated with a concentrated preparation of killer toxin for set periods of time, 0, 15, 30, 45, 60 and 90 minutes, before a sample was removed and incubated with MTT. The absorbance of the resultant supernatants was measured and the percentage reduction in viability of the sensitive cells calculated at each stage.

Figure 5.13 displayed that there was approximately a 60% reduction in viability of the sensitive strain after a 15 minute exposure to the killer toxin. There was no further conversion of MTT to MTT-Formazan and, therefore, no further reduction in cell viability, after an exposure time of 60 minutes.

(e) It was hoped that this method could be used as a rapid and accurate assay of toxin activity and that it would be insensitive to the presence of salt in fractions eluted from FPLC columns. FPLC buffers, 20 mM bis TRIS propane, pH 6.5 (buffer A) and 20 mM bis TRIS propane + 1.0 M NaCl, pH 6.5 (buffer B) were prepared and combined to produce solutions of 0-100% buffer B. A concentrated preparation of killer toxin was diluted 1:5 in these solutions and their effect on the conversion of MTT to MTT-Formazan by cells of the sensitive strain *C.glabrata*, were investigated with respect to toxin-free buffer controls.

Figure 5.12 - Comparison of the MTT-colourimetric assay of toxin activity with traditional plating techniques for monitoring sensitive cell viability. *C.glabrata* (S-388) ($1.2x10^8$ cells/ml) was exposed to different concentrations of killer toxin for 90 minutes at 20^oC.

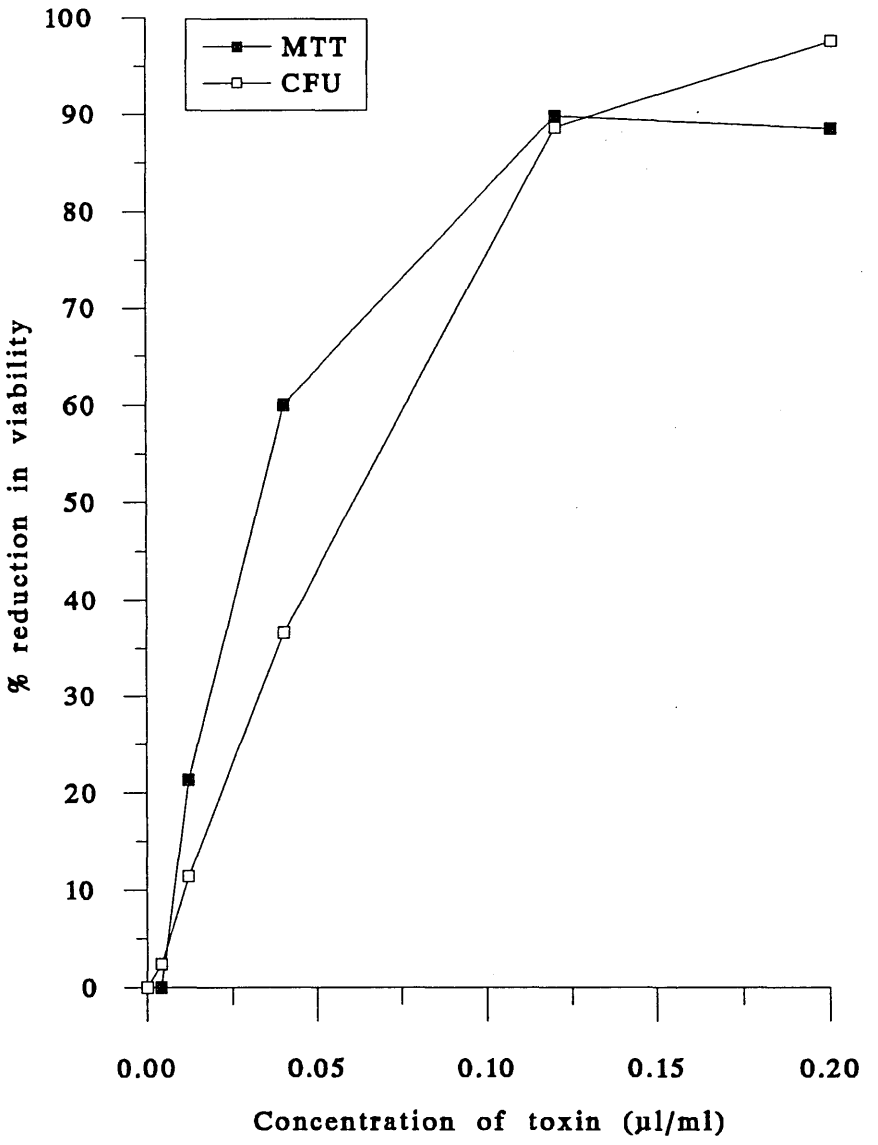


Figure 5.13 - Time-course to investigate the relationship between the period of toxin exposure and sensitive cell viability. Cells of the indicator strain *C.glabrata* (S-388) were exposed to killer toxin of the yeast *W.mrakii* (K-500). At specific intervals samples were removed and the percentage reduction in viable cells determined with respect to a toxin-free control using the MTT-colourimetric assay.

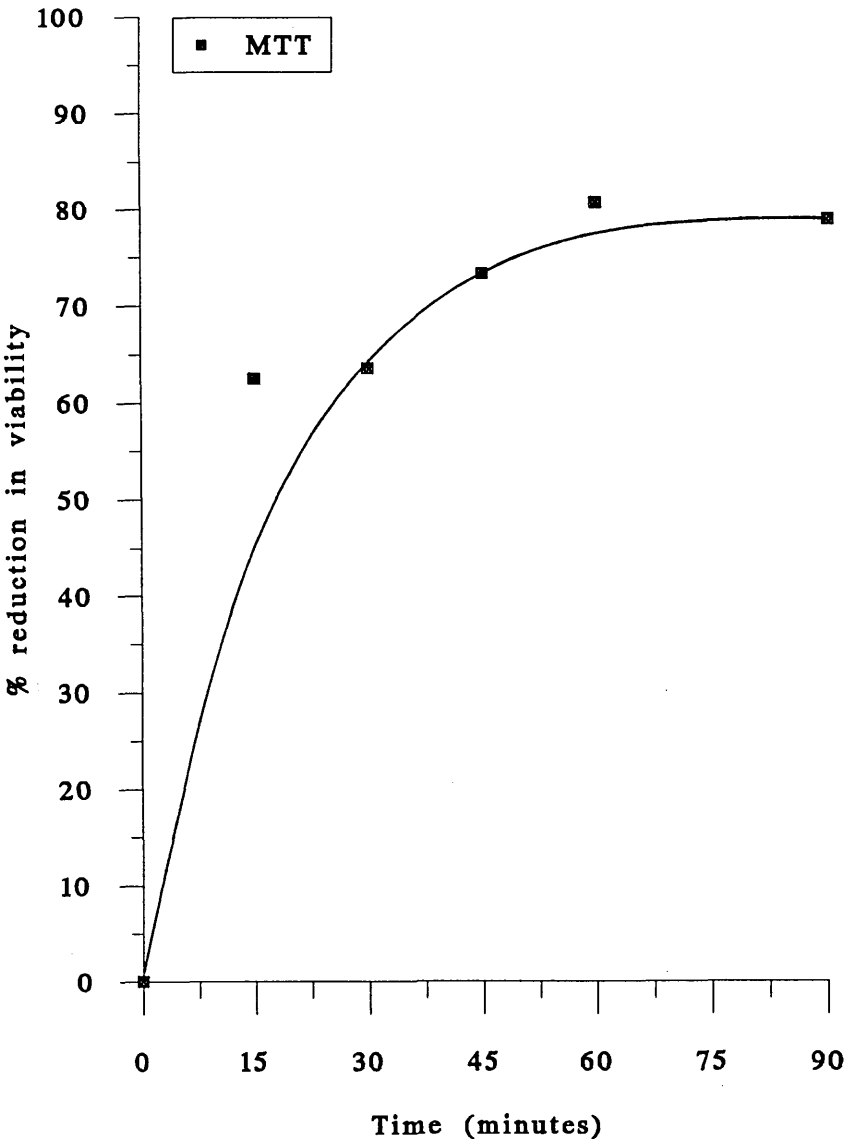


Table 5.4 - Effect of FPLC buffers on the MTT-colourimetric assay of toxin activity. Concentrated toxin was diluted in a range of buffers comprising buffer A, 20 mM bis TRIS propane, pH 6.5, and buffer B, 20 mM bis TRIS propane + 1.0 M NaCl. Residual toxin activity was measured as the reduction in viability of a sensitive strain , *C.glabrata* (S-388), with respect to toxin-free buffer controls.

Buffer Solutions	Absorbance (570 nm) + Toxin	Absorbance (570 nm) - Toxin	% Reduction in Viability
100% Buffer A	0.232	0.464	50.0
10% Buffer B	0.210	0.502	58.8
20% Buffer B	0.181	0.500	63.8
30% Buffer B	0.179	0.475	62.3
40% Buffer B	0.157	0.414	62.0
50% Buffer B	0.172	0.404	57.4
60% Buffer B	0.175	0.441	60.3
70% Buffer B	0.184	0.397	53.7
80% Buffer B	0.197	0.403	51.1
90% Buffer B	0.157	0.370	57.6
100% Buffer B	0.156	0.325	52.0

Table 5.4 shows the amount of MTT-Formazan produced in terms of absorbance readings at 570 nm, and the effect of each solution on sensitive cell viability was calculated. It was possible to detect toxin activity in the test solutions as reductions in absorbance when compared to toxin-free control solutions. The killer toxin produced a consistent 50-64% reduction in sensitive cell viability in the presence of increased concentrations of salt in the buffers.

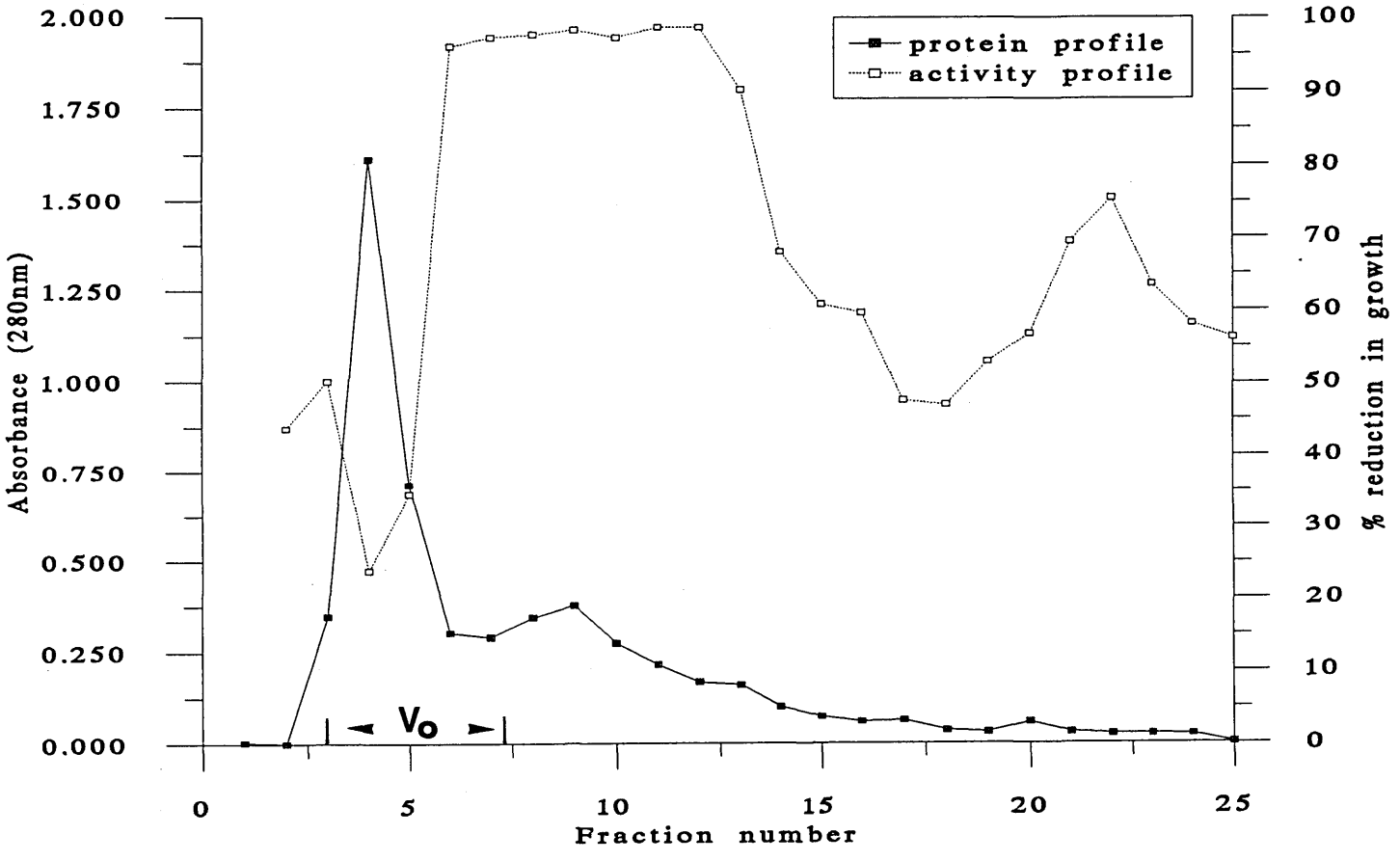
5.9 Analysis of *W.mrakii* (K-500) Killer Toxin Using Gel Filtration Chromatography

(a) Preliminary analysis of the toxin using PhastSystem SDS-Page suggested that the molecular weight of the killer factor released by *W.mrakii* (K-500) during growth was approximately 16 kDa. To 'clean up' the toxin preparation, by removal of salts and low molecular weight material, it was proposed to use a small (9 ml bed volume), commercial, pre-packed column containing G-25 Sephadex (fractionation range 1000-5000 Da). The column was prepared as described in section 2.10.2 prior to the application of crude toxin (1 ml of 100 mg/ml dry weight of lyophilisate). The sample was eluted with distilled water and 1 ml fractions were collected. The protein content of these fractions was determined by measurement of absorbance at 280 nm and 100 µl amounts were assayed for toxin activity, against *C.glabrata* (S-388), using a microtitre assay (section 2.6.3).

The elution and activity profiles obtained from this separation are represented in Figure 5.14. Two major proteins were separated on the column. Protein (A) (fractions 3-6), eluted at the void volume of the column, was associated with a relatively low activity, only producing a 24% reduction in growth of the sensitive strain. However, the second smaller protein (B) (fractions 7-13) displayed very high killing activity, with a 98% reduction in growth of the indicator strain.

(b) A second separation was performed using a larger, prepared Sephadex G-25 column (70 ml bed volume). A more concentrated sample of toxin (1 ml of 200 mg/ml dry weight) was loaded onto the column and eluted with distilled water. The collected fractions were assayed for both protein content and toxin activity.

Figure 5.14 - Elution profile of *W.mrakii* (K-500) killer toxin on a commercial pre-packed Sephadex G-25 column (9 ml bed volume). Toxin (100 mg/ml dry weight) was eluted with distilled water and the protein profile of the collected fractions (1 ml) measured at 280 nm. Toxin activity was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.



A much higher resolution of separation was achieved with the larger column (Figure 5.15) but the profiles indicated that the active protein (B) was eluted at the same stage of the separation. However, with the increase in the bed volume of the column, dilution of toxin activity occurred during the separation so it became necessary to freeze-dry and concentrate samples 10-fold prior to assay.

(c) A third separation was achieved using a prepared Sephadex G-25 column of 700ml bed volume. The crude toxin preparation (10 ml of 276 mg/ml dry weight) was loaded onto the column and 5 ml fractions were collected after elution with distilled water. Samples of each fraction were concentrated 100-fold and subsequently assayed for activity.

The larger column enhanced the resolution of the separation of the proteinaceous components of the killer factor. Strong killing activity (approximately 100% growth inhibition of the sensitive strain) was not directly attributed to any one of the separated proteins (A-C) but appeared to lie between the doublet (B and C) eluted in fractions 80-89. This separation was repeated several times and a consistent and reproducible activity and protein profile (Figure 5.16) was achieved.

(d) Samples of the active fractions (80-89) from the latter separation were pooled, freeze-dried and concentrated 40-fold. The activity of this preparation was confirmed by microtitre assay. A 750 µl sample was loaded onto a P2 Biogel column (100 ml bed volume), which has a fractionation range of 100-1800 Da, and eluted with distilled water. One ml fractions were collected and assayed for activity.

Figure 5.17 shows the elution profile obtained and the separation of four components (A-D) was achieved. Consecutive fractions were pooled and concentrated 10-fold prior to assay. The activity profile determined suggested that the two peaks eluted near the void volume of the column possessed killing activity.

A second separation using this column yielded the same profiles and two pairs of fractions, 35+36 (X) and 37+38 (Y), were combined for measurement of a UV spectra. The results in Figure 5.18 suggested that the wavelength of maximum absorption (λ_{max}) of these fractions was 257 nm.

Figure 5.15 - Elution profile of *W.mrakii* (K-500) killer toxin on a prepared Sephadex G-25 column (70 ml bed volume). Toxin (200 mg/ml dry weight) was eluted with distilled water and the protein profile of the collected fractions (1 ml) measured at 280 nm. Toxin activity was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.

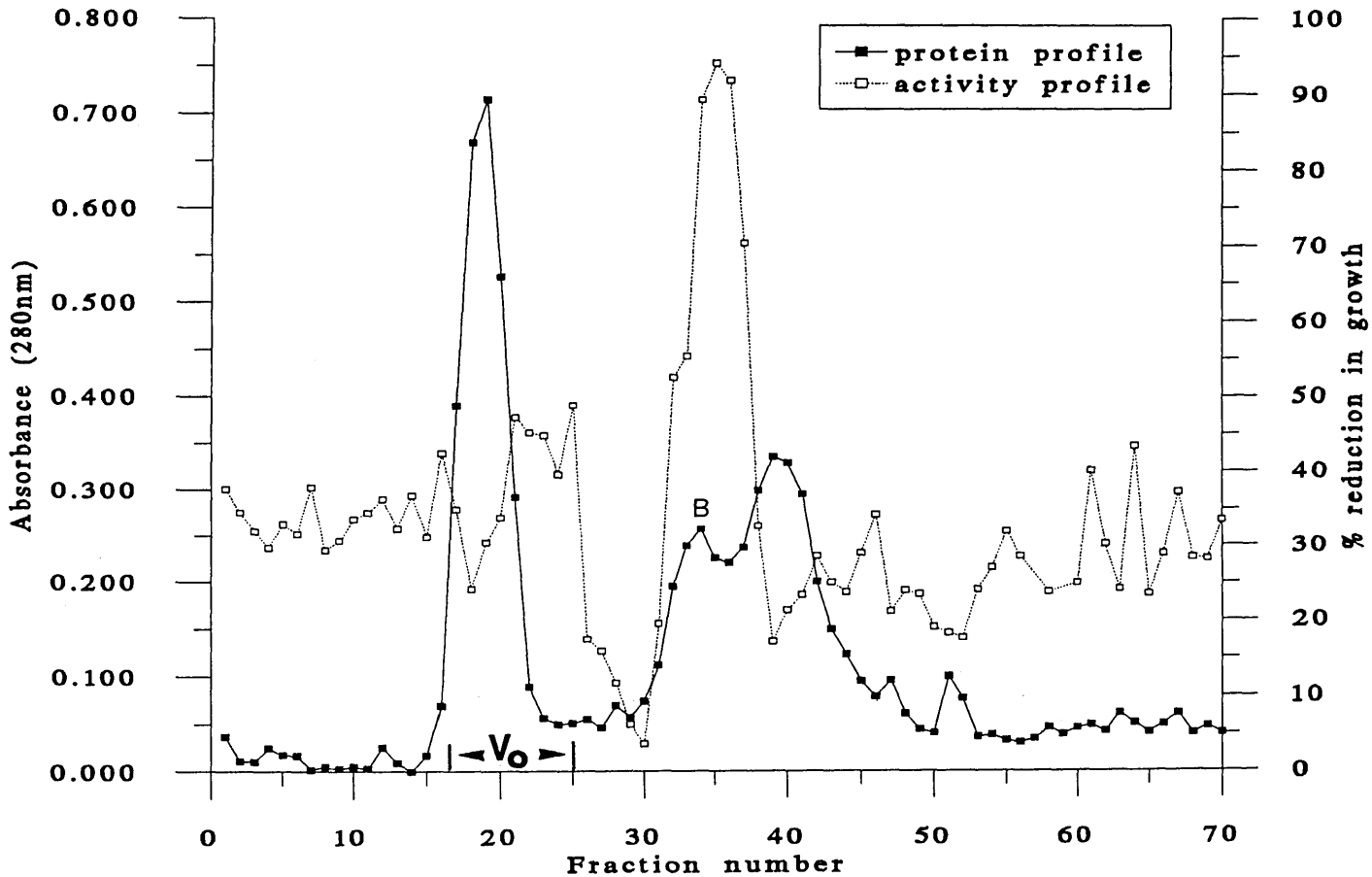


Figure 5.16 - Elution profile of *W.mrakii* (K-500) killer toxin on a prepared Sephadex G-25 column (700 ml bed volume). Toxin (276 mg/ml dry weight) was eluted with distilled water and the protein profile of the collected fractions (5 ml) measured at 280 nm. Toxin activity was measured against the indicator strain *C.glabrata* (S-388) using a microtitre assay.

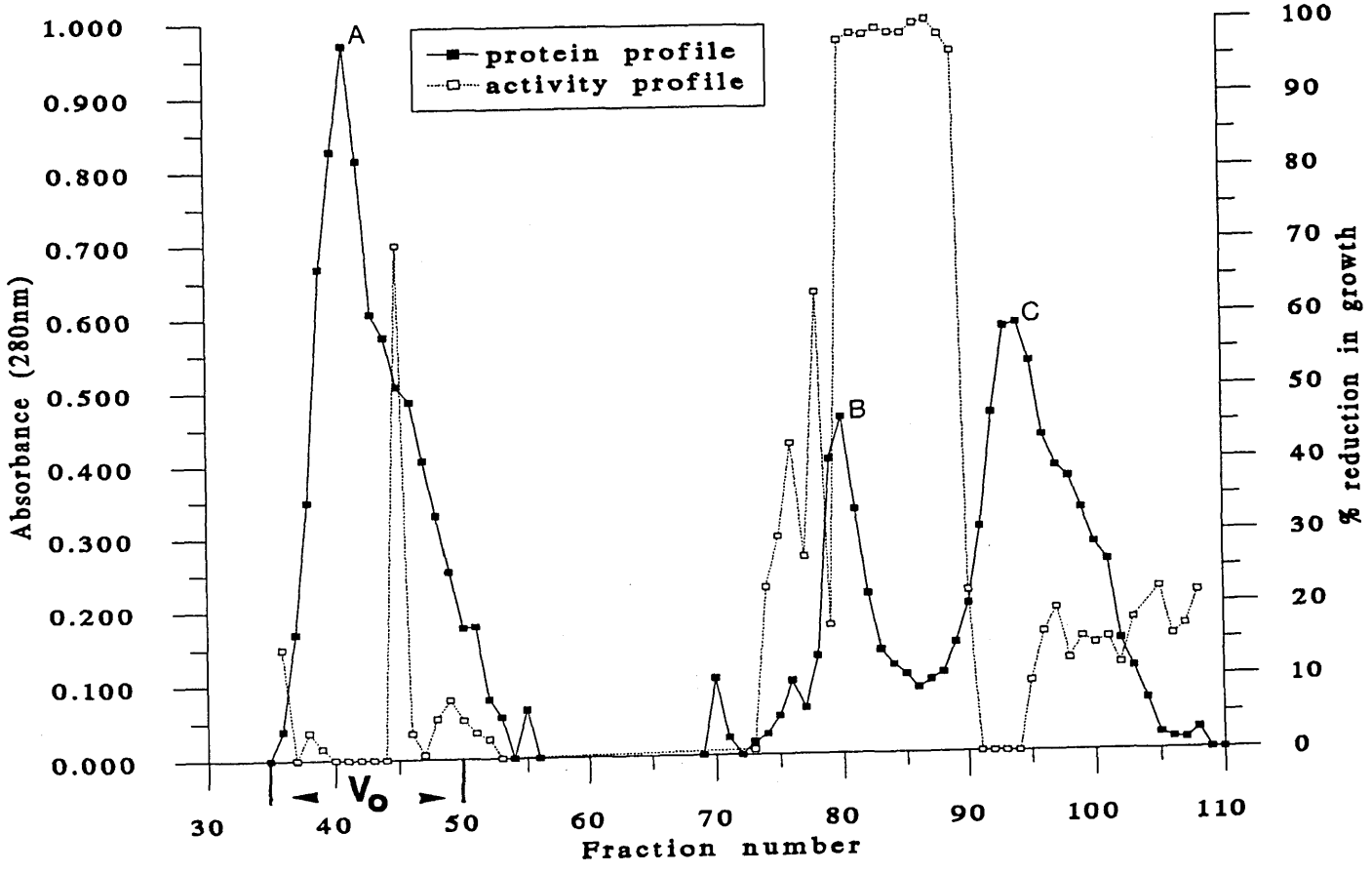


Figure 5.17 - Elution profile of *W.mrakii* (K-500) killer toxin on a prepared P2 Biogel column (45ml bed volume). Active fractions 80-89 (from separation in Figure 5.16) were pooled and concentrated 40-fold. 750 µl was applied to the column and eluted with distilled water. Protein content of the fractions was measured at 280 nm and toxin activity by microtitre assay against *C.glabrata* (S-388).

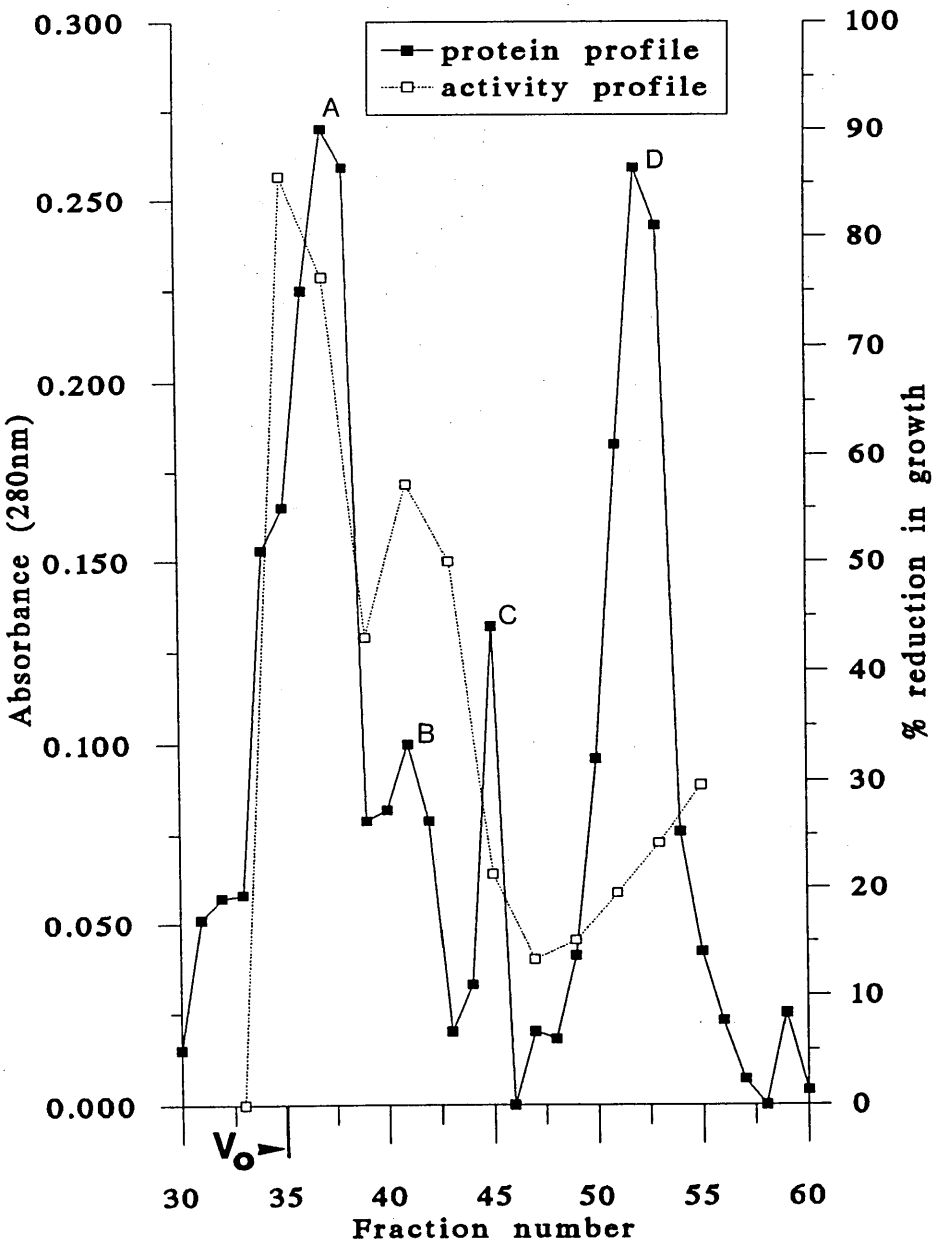
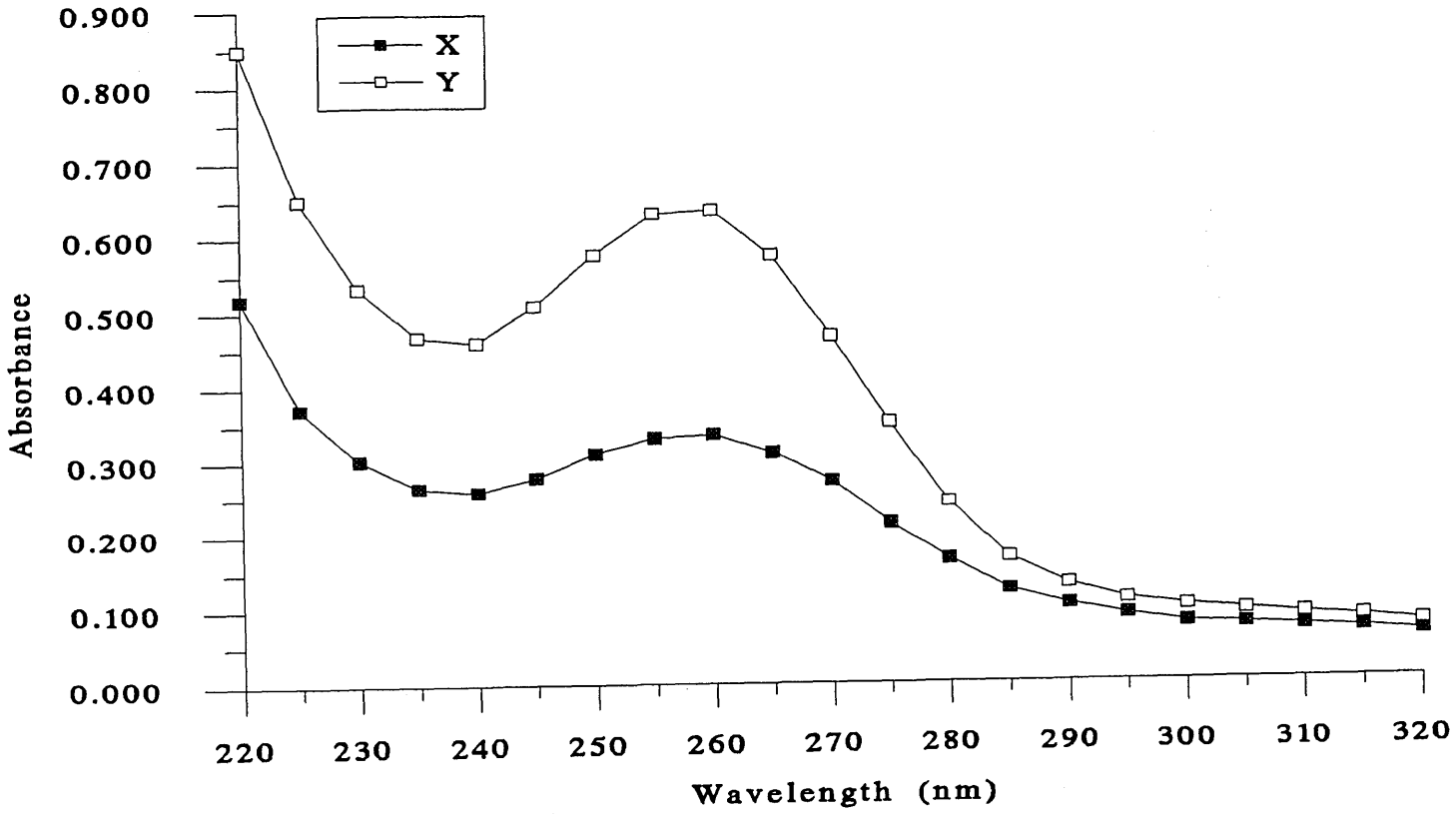


Figure 5.18 - UV spectra of combined fractions eluted from the P2 Biogel separation of *W.mrakii* (K-500) killer toxin. Fractions 35+36 (X) and 37+38 (Y) were used to determine the UV spectra of eluted samples over the range of wavelengths 220-320 nm.



(e) A 10 ml sample of a concentrated crude toxin preparation was applied to the largest G-25 Sephadex column and was eluted with distilled water. The activity present in the initial sample and that in the eluted fractions was assessed using a microtitre bioassay. Table 5.5 shows the total units of activity applied to, and recovered from the column. 2500 units of activity where present in the initial preparation and 1000 units were applied to the column. A direct assay for activity of 100 µl amounts from each of the eluted fractions did not conclusively detect toxin activity, however, there were traces of activity in fraction 69. The fractions were, therefore, reassayed after a 10-fold concentration and the results highlighted toxin activity in fractions 67-75 (Figure 5.19). Although a different profile was obtained, the volume of eluant required to remove active fractions (400-450 ml), was consistant with previous separations. The total toxin activity present in these fractions (54 units) was, however, only a small percentage (5%) of the activity originally loaded.

(f) The binding of the active component of the toxin to the Sephadex beads during separation by gel filtration chromatography was also investigated. The same toxin preparation from the previous study was used and the procedure followed is outlined in section 2.10.2.1. The toxin solution (A1) was incubated with a small amount of pre-swollen Sephadex G-25, the slurry was then centrifuged and the resultant supernatant assayed for activity (A2). The Sephadex was washed twice with distilled water (A3 and A4) and once with 0.1 M NaCl (A5). The effect of 0.1 M NaCl on the assay system was also assessed. Toxin activity in 10-100 µl amounts of each solution was measured using a microtitre bioassay.

From the results in Figure 5.20 it would appear that approximately 60% of the toxin activity was recovered in the initial centrifugation. No activity was recovered from washing the Sephadex with distilled water and only negligble amounts from the salt washing. 0.1 M NaCl did not inhibit growth of the indicator strain.

Table 5.5 - Assessment of toxin activity during fermentation, processing and gel filtration chromatography. Killing activity was assayed against *C.glabrata* (S-388) using a microtitre bioassay.

Toxin Preparation	Volume (ml)	Equivalent Unit[a] of Activity (µl)	Total Activity (units)	Loss of[b] Activity (%)
Cell-free Supernatant Freeze-dried and Concentrated (x20)	25	10	2500	0
Sample Loaded onto G-25 Sephadex Gel Filtration Column	10	10	1000	0
Fractions Collected (6 ml) ; 67-75 Showed Toxin Activity	54	1000	54	95

a - 1 unit of activity is defined as that volume which results in approximately 100% reduction in growth of the indicator strain in a microtitre assay.

b - the percentage loss of activity is relative to the previous step in the table.

Figure 5.19 - Elution profile of *W.mrakii* (K-500) killer toxin on a prepared Sephadex G-25 column (700 ml bed volume). Toxin (concentrated 20-fold) was eluted with distilled water and the protein profile of the collected fractions (6 ml) measured at 280 nm. Toxin activity was measured against *C.glabrata* (S-388) using a microtitre assay.

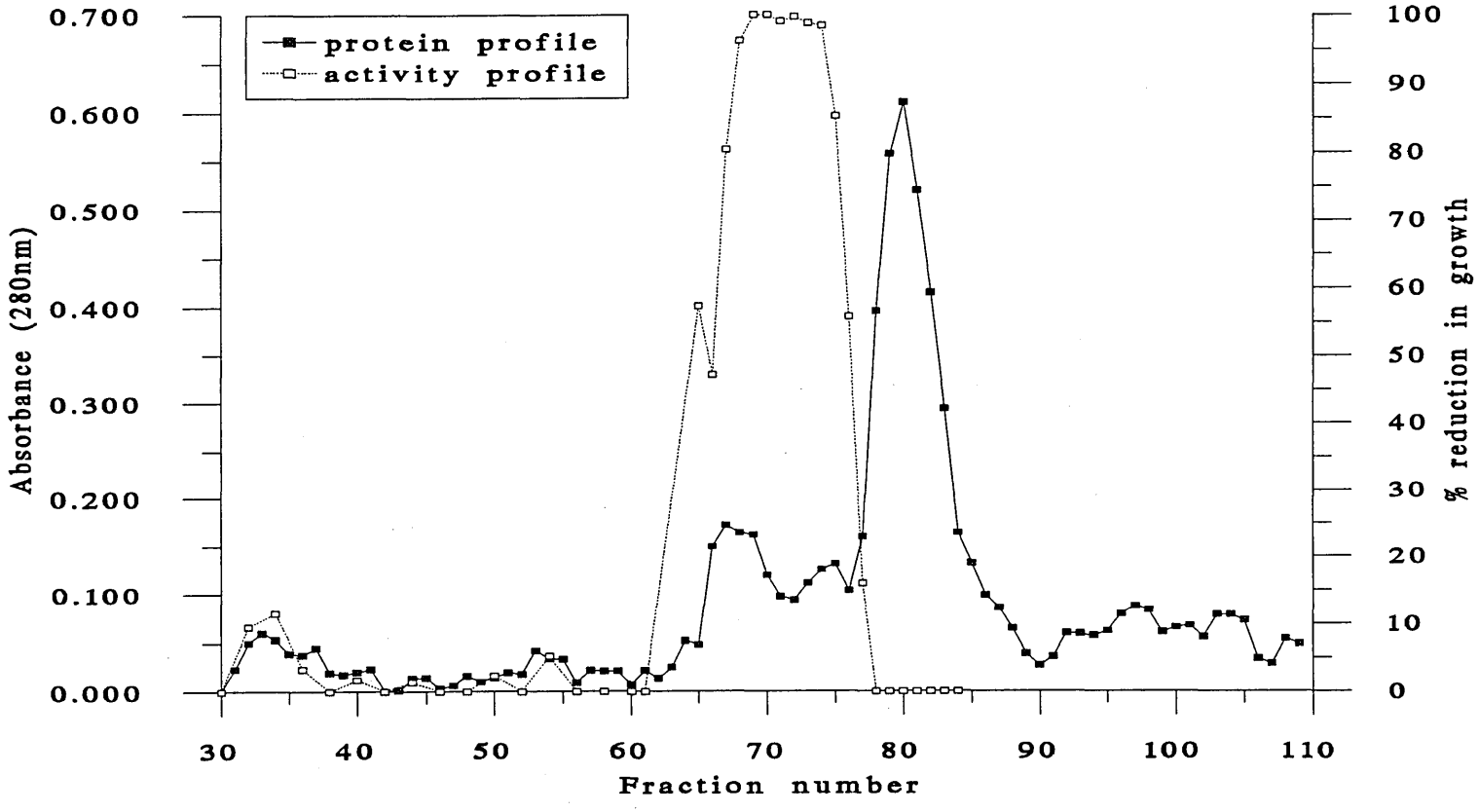
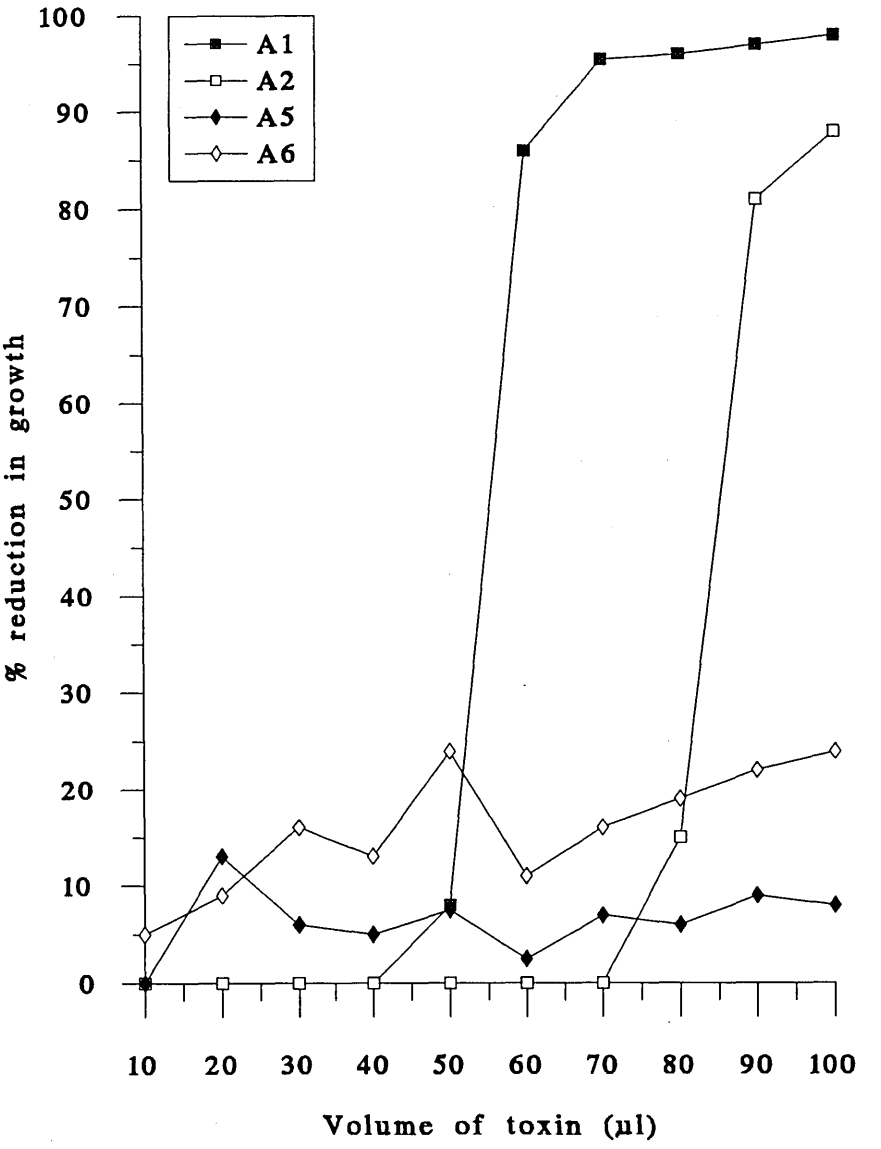


Figure 5.20 - Binding of *W.mrakii* (K-500) killer factor to G-25 Sephadex. Killer toxin (A1) was incubated with G-25 Sephadex for 1 hour at 20^{0}C and removed by centrifugation. Residual toxin activity (A2) in the supernatant, and in subsequent water and salt washes (A5), was measured by microtitre assay against *C.glabrata* (S-388). The effect of 0.1 M NaCl (A6) on the assay system was also investigated.



5.10 Discussion

It was the purpose of the work reported in this chapter to characterise the killer toxin of *W.mrakii* (K-500) in terms of its biochemical properties and to attempt a purification of the killer factor produced using the techniques of FPLC and gel filtration chromatography.

It was found that the killer toxin produced by *W.mrakii* K-500 was stable up to 24 hours at $4^{o}C$ and $18^{o}C$, however, the inherent stability was reduced as incubation temperatures were increased. 80% of the toxin activity remained at $25^{o}C$ over the same time period, but was reduced to 5% at a temperature of $30^{o}C$. The toxin was stable at this temperature for up to 6 hours, but a steady decrease in activity followed thereafter. At $37^{o}C$, toxin activity was reduced after 5 hours and was completely inactivated between 7 and 24 hours (Figure 5.1). The killer factor was readily inactivated at temperatures exceeding $50^{o}C$ and showed no resistance to high temperature treatment. The optimum pH of the killing activity of the toxin in an agar diffusion bioassay was found to be pH 3.5 (Table 5.1). However, the killing action of the toxin was reduced at pH's up to pH 4.5, and inactivation occurred at pH 5.0. It cannot be discounted, however, that the pH of the agar system directly affected the growth of the sensitive strain, as can be seen by the failure of S-388 to grow at pH 7.0 and above. It may be that pH 3.5 was the optimum growth pH for the sensitive *Candida* strains or the pH of the surrounding medium affected receptor development on the sensitive yeast cell. Using a microtitre assay of toxin activity it was found that the killer factor was stable between pH 2.4 and 4.0 over a period of 18 hours. The killer factor was, however, rapidly inactivated at pH's above this (Figure 5.2). Therefore, it is feasible that the optimum killing activity does in fact lie within this range at pH 3.5. The range of toxin stability may be extended if preparations are maintained at specific values for shorter periods of time.

The information obtained further distinguished the killer factor produced by K-500 from that produced by the strain LKB 169. A previous report by Ashida *et al.* (1983), stated that the killer toxin produced by a strain of *W.mrakii*, LKB 169, was stable to boiling at $100^{o}C$ for 3 minutes when maintained at pH 4.0, and was stable over the range pH 2.0-11.0. No mention was made of the stability of the toxin at reduced temperatures.

Extreme variation in the temperature and pH profiles of toxin action exists between killer yeasts from different genera and between strains of the same species. Woods *et al.* (1974) reported that a toxin of the killer yeast *S.cerevisiae* was stable between pH 4.6-4.8 and was totally inactivated after incubation at 32^{o}C for 30 minutes. Shimizu *et al.* (1985), however, observed that the toxin from another *S.cerevisiae* killer yeast was stable between pH 2.9-4.9 and at a temperature of 30^{o}C for several days, but was inactivated at 48^{o}C after 10 minutes. The numerous reports agree, however, that the killer factors produced are generally inactivated above pH 5.0 and are labile to temperatures exceeding 45^{o}C. Exceptions to this include the toxin of LKB 169 (Ashida *et al.*, 1983) and a chromosomally-inherited toxin in *S.cerevisiae* (Goto *et al.*, 1990) which was stable to pH 7.5. The potential of toxin preparations to be used directly as novel therapeutics may, therefore, be limited because of their narrow range of stabilities. However, the differences observed in the physical properties further indicates that structurally distinct killer factors are produced by different killer yeasts, a property which could be exploited further in their classification.

The effect of the proteolytic enzymes pepsin and pronase on killer toxin activity in two strains of *W.mrakii*, K-500 and K-LKB, was investigated. The toxins were incubated with each enzyme or a suitable control, which assessed the effect of the enzyme's diluent, distilled water (pepsin) or $CaCl_2$ (pronase), on toxin activity. Boiled controls also insured that any toxin inactivation produced was dependent on the catalytic activity of the enzyme.

The results (Figure 5.5) suggested that both toxins were insensitive to the action of pronase and pepsin. This is similar to the findings of Young and Yagui (1978), however, their study indicated that the killer factor of *W.mrakii* K-500 was also sensitive to native papain at a pH of 4.2 or pH 4.6. Components present within the concentrated culture supernatants may protect the toxin from proteolytic digestion and this would explain the insensitivity of the toxin preparations to pepsin and pronase. Alternatively, the toxin may contain a carbohydrate moeity, as in the *K.lactis* toxin (Stark *et al.*, 1990), which interfered with proteolytic digestion. However, in all likelihood, the amino acid composition of the killer factors are such that there were insufficient, or incorrect, sites

for proteolytic cleavage by the two enzymes investigated. High temperature treatment (boiling for 10 minutes) destroyed all activity in the killer factor produced by strain K-500, however, 60-80% of the activity of the killer toxin of K-LKB remained. This stability to high temperatures was suggested by the earlier work of Ashida *et al.* (1983).

Initially killer toxin activity was detected in the retentate fraction, greater than 10 kDa, following Amicon ultrafiltration of the extracellular medium (see section 4.3). The media used and the subsequent processing of cultures, suggested that the amount of extraneous proteins and polypeptides present in the retentate would be minimal. Therefore, it was hoped that analysis of this fraction would yield valuable information concerning the killer factor of *W.mrakii* (K-500).

The Pharmacia PhastSystem allowed a fast, high resolution and reproducible electrophoresis and isoelectric focussing to be performed on proteinaceous material. A microprocessor within the system regulated all parameters during the separation and development of gels and results were available within 2 hours. It was hoped that the system would allow a rapid, preliminary assessment of some of the characteristics of *W.mrakii* (K-500).

IEF is a high resolution method in which proteins are separated in the presence of a continuous pH gradient. Under these conditions, the proteins migrate according to their charges until they reach the pH values at which they have no charge *i.e.* their isoelectric points (pI). The proteins achieve a steady state of zero migration and they are concentrated and focussed into narrow zones (Dunn, 1989). The estimated isoelectric points of the components of the crude toxin lay between pH 4.35 and pH 5.80, which was consistent with the toxin of *W.mrakii* being acidic (Table 5.3). This also explained the change in colour from blue to yellow when the toxin was prepared in boiling mix for SDS-PAGE analysis. A protein with an isoelectric point of 6.15 was present in both the toxin sample and the media control, therefore, it was assumed that this did not contribute to the action of the toxin. Five other proteins with distinct isoelectric points were visualised following silver staining, but it was not determined which, if any, of these bands possessed killer activity. No bands were visible after high temperature treatment which suggested that the proteins were denatured and their physical structure irreversibly damaged.

Sodium dodecyl sulphate, SDS, an ionic detergent is an effective solubilising agent for a wide range of proteins. The detergent binds to, and masks, the intrinsic charge of the polypeptide chains, so the net charge per unit mass becomes constant. Therefore, subsequent electrophoretic separation is dependent only on molecular size (Dunn, 1989). A weakly-stained band was detected on the silver-stained PhastGel, with an apparent molecular weight of 16 kDa. It was suprising that only a single band was present in such a crude preparation, especially when previous IEF analysis highlighted the presence of five protein bands, however, they may have been present in amounts below which they could be detected. Further attempts to detect protein bands in toxin preparations, by electrophoresis on Mini-Gels or PhastSystem gels, were unsuccessful. This suggested that the band previously visualised was not in fact toxin protein. If present, small polypeptides and peptides, generally less than 10 kDa, cannot be separated by the traditional SDS-PAGE system. The small molecules form SDS-protein complexes of the same dimension and charge which migrate rapidly during electrophoresis and fail to be resolved or distinguished from the dye front. However, addition of urea, a solute which reduces the size of detergent micelles, can overcome this problem (Swank and Munkres, 1971). Subsequent work suggested that the molecular weight of the toxin was in fact smaller than 5 kDa (see section 5.9), therefore, use of this modified system may have allowed separation of the toxin proteins. Sawant *et al.* (1989) suggested that the appearance of unusually large molecules during electrophoresis, and the shift of peaks during purification of the killer toxin of *P.anomala* by ion-exchange chromatography, was a result of the aggregation of toxin molecules. Therefore, the appearance of a band corresponding to a size of approximately 16 kDa, may have been due to the aggregation of several smaller toxin molecules.

The separation of components present in crude toxin preparations was necessary to further investigate the properties of the secreted killer factor of *W.mrakii* (K-500). Electrophoretic patterns suggested a simple mixture of proteins was present in partially purified extracts, therefore, immediate use of Fast Protein Liquid Chromatography (FPLC) appeared to be the most profitable approach.

FPLC was developed for a high performance separation of biomolecules such as proteins, peptides and amino acids. The separations are rapid, of a high resolution and

have full biocompatibility for the maximum recovery of biological activity. A Mono Q ion-exchange column was used for the separation of the components of *W.mrakii* (K-500) killer toxin. Mono Q is an anion exchange column carrying a positively charged ligand which will bind and exchange negatively charged molecules. When a protein is at its isoelectric point (pI), it possesses no net overall charge, at pH's above its pI it is negatively charged and pH's below it is positively charged. The column was equilibrated with a buffer of specific pH before a sample of killer toxin was loaded. Components of the crude preparation passed over the column if positively charged, or they were bound to the column by varying degrees depending on the overall charge. Bound proteins were removed by a linear gradient of buffered NaCl, negatively charged proteins were replaced by Cl^- ions, and washed out in the eluant. Fractions were collected and assayed for activity.

An initial separation using 20 mM TRIS, pH 8.0 yielded four main protein peaks (Figure 5.6a). The increasing activity displayed by fractions from these peaks, A through to D (Figure 5.6b), was in all likelihood, a direct effect of the increasing salt concentrations present in the eluting buffer. Selected fractions from a blank gradient control produced increased growth inhibition of the indicator strain in a microtitre assay (data not shown). Inhibition increased as the amount of buffer B and, therefore, salt, present in the elution buffer increased. The pH of the system was much greater than the pI's determined previously for the components of the crude toxin, therefore, a negative charge would be imparted on the proteins. This would result in strong binding of the proteins to the column and their subsequent detection may have been masked by the high concentrations of Cl^- ions present in the samples. A pH 4.0 (20 mM *N*-methylpiperazine) buffer system was used and the results indicated (Figure 5.7a and 5.7b) that the active toxin was present in the unbound fraction and, at a pH below their pI, the active components carried a positive charge. The pH of the following buffer systems were raised to impart a slight negative charge on the protein, so that it bound early on the column and would be readily eluted by low levels of Cl^- ions. Use of a pH 6.0 (20 mM bis TRIS) buffer system yielded a single fraction with lethality against a sensitive strain (Figure 5.8b). A further increase to pH 6.5 (20 mM bis TRIS propane) resulted in the separation of several proteins (Figure 5.9a), however, none could be conclusively assigned toxin activity (Figure 5.9b).

Toxin activity was consistently detected in unbound material removed from the column when buffer systems between pH 4.0 and 6.0 were used. However, when the pH of the buffer system was increased to above pH 6.0, toxin activity was 'lost' from the unbound fractions. Interference in the microtitre assay, caused by increasing salt concentrations in the elution buffer, appeared to be delayed to later fractions (36-45) in buffer system 4 (Figure 5.9b). In buffer system 3 (Figure 5.8b) the 'salt effect' was observed in fractions 20 to 40. This suggested the presence of toxin protein in fractions removed from the column in the former system, however, activity could not be accurately assigned to any of the bound proteins. Repeated attempts were made to apply, and separate, components of the toxin at pH's above their isoelectric points, but no clear toxin activity was detected. It is possible, therefore, that the killer factor was inactivated by the increased pH of the buffer systems used, there was irreversible binding of active components to the column or, the activity present in the eluted fractions fell below the threshold limit of the assay system.

Due to difficulties experienced in the purification of the killer factor produced by *W.mrakii* using FPLC, an alternative method involving gel filtration chromatography was attempted. The separation of material using this technique depends on the different abilities of the sample molecules to enter pores in the gel matrix which contain the stationary phase. Very large molecules which do not have access to the stationary phase pass rapidly through the chromatographic bed. Smaller molecules which can enter the gel pores, move more slowly through the column since they spend a proportion of their time in the stationary phase. Molecules are eluted, therefore, in order of decreasing size. Each Sephadex G-type has a different molecular weight range over which molecules can be fractionated. Those species with molecular weights above the upper limit of this range are totally excluded from the gel matrix and are eluted at the void volume. The void volume in each case was determined by the passage of the indicator molecule blue dextran ($1x10^6$ Da) through the column.

Previous work had suggested that the molecular weight of the toxin was greater than 10 kDa, therefore, use of a G-25 Sephadex matrix (fractionation range 1-5 kDa) would provide a 'clean-up' step to remove any low molecular weight salts and carbohydrate from the crude toxin preparation. A molecule of known molecular weight, Ribonuclease

A (13.7 kDa), was applied to the commercial (9 ml bed volume) column to check the homogeneity of the bed and the effective elution of proteinaceous material. As expected the protein was excluded from the Sephadex matrix and was eluted at the void volume of the column in fractions 4-8. Crude toxin (1 ml of 100 mg/ml dry weight of lyophilisate) was applied to the column and two proteins were separated (Figure 5.14). A large peak (A) was eluted at the void volume and was not associated with toxin activity, however, the smaller protein peak (B) displayed lethality against the indicator strain *C.glabrata* in a microtitre assay. Protein (B) was eluted after the void volume of the column which suggested that its molecular weight fell within the fractionation range of the dextran matrix. The relatively high activity associated with fractions 20-25 was, in all likelihood, an effect of salt or low molecular weight carbohydrate present in the crude toxin preparation.

A larger column was prepared to enhance the resolution of the separation of components within the toxin sample. A more concentrated preparation (200 mg/ml dry weight) was applied to a 70 ml column of G-25 Sephadex and a very similar elution profile was achieved (Figure 5.15). Protein (A), the large molecular weight material excluded from the column, was eluted in fractions 16-22 and possessed no killing activity. Toxin activity was, however, associated with fractions 32-38. Similarly, toxin activity was associated with fractions 80-89 (Figure 5.16), when components of the toxin were separated on a 700 ml column. In both cases, toxin activity did not appear to be associated directly with a protein peak, but rather the area between the eluted doublet, (B) and (C).

It was surprising that the proteins eluted at the void volume showed no toxin activity, but the active component of the preparations was separated on the column. The molecular weight, hitherto assumed to be a minimum of 10 kDa may be as low as 5 kDa. The active fractions from the latter separation, were pooled and concentrated by freeze-drying. They were applied to a P2 Biogel (fractionation range 100-1800 Da), to confirm that the active component of the killer toxin preparation was not a large protein but a small peptide. The two protein peaks eluted at the void volume of the column (Figure 5.17) displayed killing activity, which suggested that the active component possessed a minimum molecular weight of 1800 Da. This molecular weight was significantly smaller than any other previously reported. The K1 killer toxin of

S.cerevisiae has a molecular weight of 20,658 Da (Zhu *et al.*, 1987), the glycoprotein toxin of *P.anomala* WC 65, a molecular weight of 83,300 Da (Sawant *et al.*, 1989) and *P.kluyveri* 19,000 Da (Middelbeek *et al.*, 1979). However, the smallest toxins reported to date are produced by yeast of the same genus, *W.mrakii* LKB 169, produces a toxin of 8,900 Da (Ashida *et al.*, 1983), and *W.saturnus* one of 8,500 Da (Ohta *et al.*, 1984). The size of the molecule was consistent with the killer factor acting as an ionophore on sensitive yeast strains (section 6.5). Similarly, the bacteriocins nisin and subtilin produced by *Streptococcus lactis* and *Bacillus subtilis* respectively, are low molecular weight peptides with broad-spectrum activity against Gram-positive bacteria, including many pathogenic food spoilage organisms (Hansen *et al.*, 1989).

Most proteins exhibit an absorption maximum at 280 nm which is attributable to the phenolic group of tyrosine and the indolic group of tryptophan. The UV spectra of the active fractions 35+36 (X) and 36+37 (Y), eluted at the void volume of the P2 Biogel column were measured (Figure 5.18). The absorption maximum (λ_{max}) of the active fractions was approximately 257 nm and there was very little absorption at 280 nm. This explained why the toxin activity appeared to lie between the two protein peaks separated by gel filtration chromatography. If the molecular weight of the active toxin molecule was small, approximately 15-20 amino acid residues, it was not surprising that tryptophan and tyrosine were absent, hence, the reduced λ_{max}. A similar situation to that observed was reported by Sawant *et al.* (1989) when attempting to purify the killer toxin produced by *P.anomala* WC 65. Very little absorption at 280 nm was found and a failure to detect the toxin protein by Bio-Rad dye-binding procedures led to the suggestion that hydrophobic residues in the tertiary structure of the glycoprotein were masked by a carbohydrate moeity, or there were in fact almost no hydrophobic sites.

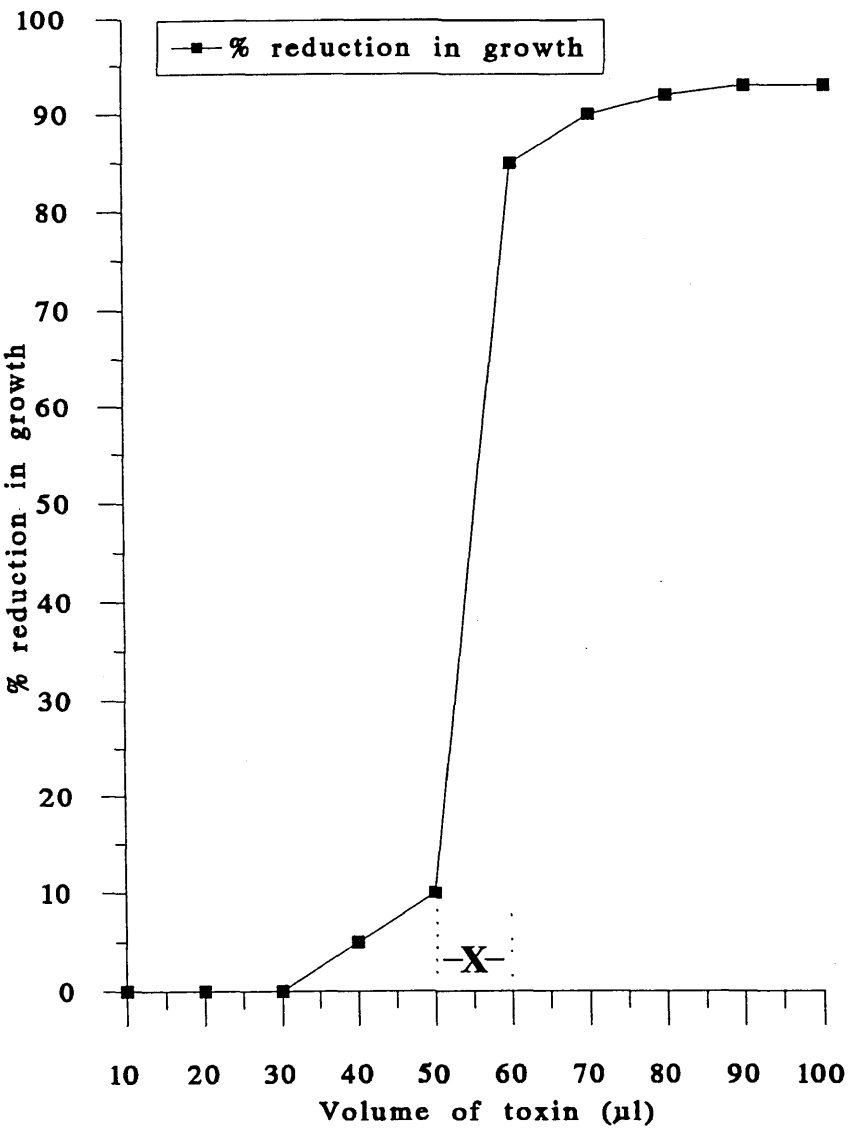
A 10 ml sample of concentrated crude toxin was applied to the largest (700 ml bed volume) column in an attempt to 'bulk up' active material for further analysis. A slightly different elution profile was obtained during this separation (Figure 5.19), however, the active protein was eluted in fractions 67-75, the same volume of eluant being required to remove the active components as in earlier separations. Total toxin activity was measured (Table 5.5) and only 5% of that originally loaded was removed. Earlier stability experiments suggested that the toxin was stable to ambient temperatures for up to 24 hours (Figure 5.1), therefore, it was unlikely that toxin activity was lost as a

result of the separation. The killer toxin of *S.cerevisiae* was reported (Woods and Bevan, 1968) to exhibit a tendency to be adsorbed to Sephadex during purification, therefore, it was proposed to investigate the potential adsorption of *W.mrakii* killer toxin to Sephadex G-25.

A sample of *W.mrakii* killer toxin was incubated with pre-swollen Sephadex and then the slurry was centrifuged to remove the dextran matrix. Toxin activity in the resultant supernatant was measured (A2) and it was found that 60% of the initial activity (A1) was recovered at this stage. Negligible levels of toxin activity were detected following distilled water washes of the Sephadex, and only low levels following a salt wash (A5). 0.1 M NaCl was found not to significantly affect the indicator strain used in the microtitre assay (A6) (Figure 5.20). The results suggested that the remaining 40% of toxin activity was bound to the Sephadex. However, it was possible that following centrifugation it was present within the bed volume of the dextran matrix. Consequently, if the toxin was removed during the washing stages it may have been diluted to the extent that its activity fell below the threshold of the microtitre assay. Although the results of this experiment were inconclusive, they do suggest that binding of toxin molecules to the chromatographic bed alone would not explain the extensive loss of toxin activity observed during gel filtration. It was possible that there was a dissociation of the active components of the toxin during separation and this resulted in a reduction in the activity recovered from the column. Complementation studies were conducted where all fractions, or groups of fractions, eluted from the column were combined and assayed for toxin activity. No lethality against the indicator strain *C.glabrata* (S-388) was detected (data not shown).

The microtitre assay used to assess the toxin activity of culture supernatants, or partially purified material, relied on growth or no growth of the seeded indicator strain. The assay was sensitive to relatively low concentrations of killer factor present in culture broths, however, it displayed a marked 'threshold limit' below which no activity was detectable (Figure 5.21). This threshold covered a very narrow range (**x**) and only a small increase in toxin concentration was necessary to produce extensive inhibition of the sensitive strain. This suggested that a critical number of toxin molecules were required to bind to, or react with, the sensitive strain to produce a toxic effect. Dilution of the toxin activity

Figure 5.21 - Theoretical plot to show the 'threshold limit' of the growth reduction microtitre assay.



to below the threshold limit during separation by gel filtration chromatography led to inconclusive results. Concentration of the fractions was, therefore, necessary to detect lethality in the sensitive strain. The simplicity of the growth reduction microtitre assay also led to its sensitivity to salt concentrations in FPLC buffers, thus toxin activity was 'masked' by the salt present in the eluted fractions. It was hoped to develop an assay system which was sensitive to low toxin concentrations and was unaffected by salt in the buffer systems employed in the separation techniques.

Many biological assays require the measurement of surviving or proliferating cells. This can be achieved by several methods including the counting of cells which include or exclude a dye, measurement of the incorporation or release of a radioactive label or a spectrophotometric assay which relies on a distinct colour change. Ideally a colourimetric assay for living cells will utilise a substrate that is modified to a coloured product. Tetrazolium salts measure the activity of dehydrogenase enzymes in living cells (Slater *et al.*, 1963), the tetrazolium ring is cleaved only by active mitochondria. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] has been used to develop a quantitative assay for mammalian cell survival, cytotoxicity and proliferation (Mosmann, 1983), to measure candidacidal activity of mouse neutrophils (Ashman, 1986) and more recently in an assay system to assess the biological activities of toxins produced by the plant pathogen *Alternaria alternata* (Lee *et al.*, 1991). Based on the assumption that the killer toxin of *W.mrakii* affects sensitive cell viability (see section 6.1), MTT could be used to develop a colourimetric assay system which quantitatively assessed toxin-treated cells.

The growth phase of the indicator strain *C.glabrata* was important to the success of the assay. Log-phase cells, presumably with increased mitochondrial activity, were necessary for the reduction of the MTT-dye to MTT-Formazan. Stationary-phase cells had no effect on the cleavage of MTT to the blue end-product (data not shown). The time course conducted (Figure 5.13) suggested that a 60-90 minute incubation period of the toxin with the sensitive cells was sufficient for maximum reduction of MTT by *Candida* blastospores. Based on this result, a 90 minute incubation system was used as the standard in following experiments. The MTT-Formazan reaction product is only partially soluble, therefore, acidified isopropanol was used to fully dissolve the product

and produce a homogenous solution for absorbance measurements to be made (Mosmann, 1983).

Figure 5.10 showed that the conversion of the dye, and measured absorbance, increased with cell numbers and that dead yeast cells produced no reduction of MTT. The inactivation of the heat-killed cells was verified by a failure to grow on SDA plates. Successive experiments showed that a minimum of $1x10^7$ cells/ml were also required for significant conversion of the dye. This ability to distinguish between live and dead, hence, viable cells was the basis for the assay system which developed.

After exposure to *W.mrakii* killer toxin the sensitive cells were reduced in their ability to produce MTT-Formazan (Figure 5.11). At high toxin concentrations (0.20 µl/ml) there was very little conversion of MTT to MTT-Formazan, hence, the low absorbance value of 0.059. At lower toxin concentrations (0.008 µl/ml), extensive conversion of the dye occurred (absorbance = 0.465), indicating that mitochondrial enzyme activity was intact in these cells.

To confirm that *W.mrakii* killer toxin was affecting sensitive cell viability and hence, MTT-dye conversion, a comparison was made between the colourimetric assay and traditional plating techniques (Figure 5.12). The reduction in viability of the sensitive cells with respect to increasing toxin concentrations was measured, and the subsequent reduction in the numbers of colonies produced, correlated with the decreased conversion of MTT to MTT-Formazan.

It was important also to determine if the assay was applicable to the detection of killer toxin activity in solutions which contained high levels of salt *i.e.* eluted fractions from FPLC separations. A toxin preparation was diluted in solutions containing a range of concentrations of the FPLC buffers 20 mM bis TRIS propane (buffer A) and 20 mM bis TRIS propane + 1.0 M NaCl (buffer B). Although an increase in the amount of buffer B, hence, the amount of salt present in the solutions, reduced the conversion of MTT to MTT-Formazan, residual levels of toxin activity were still detectable. Over the range of 0-100% buffer B a 50-64% reduction in viability of a sensitive strain indicated that the assay was sensitive to the same number of toxin molecules in each case.

Concentrated toxin preparations were used to develop the assay, however, results suggested that toxin activity could be detected directly from cell-free supernatants at the completion of fermentation of the killer yeast, but it may not be sensitive to the initially

low levels of toxin produced. The assay also provided a rapid system (5 hours from the initial incubation) for analysis of samples when compared to agar diffusion bioassays (2-3 days) and microtitre assays (24 hours), which is an important consideration in downstream processing and purification. These results confirmed the potential of the dye-reduction assay as a means of quantitatively assessing the action of killer toxins on sensitive strains of *Candida*.

5.11 Conclusions

W.mrakii (K-500) killer toxin was stable for several hours at temperatures up to 37^{o}C, but was readily inactivated within minutes at temperatures exceeding 50^{o}C. The killer factor was stable only over a narrow range of pH 2.4-4.0 and above pH 5.0, killing activity in agar bioassay systems was lost. The toxin was insensitive to the action of the proteolytic enzymes pepsin and pronase, however, further evidence suggested that the toxin molecule was proteinaceous. The isoelectric points of components of the crude toxin, pI 4.35-5.80, were consistent with the toxin being acidic.

Purification attempts using FPLC ion-exchange techniques achieved separation of the components of the toxin preparation, however, it was not possible to accurately assign activity to the fractions when eluted by the linear salt gradient. Gel filtration of crude toxin using Sephadex G-25 and P2 Biogel columns suggested that the molecular weight of the killer factor was between 1800-5000 Da. The wavelength of maximum absorption of the active fractions was found to be 257 nm, which was consistent with the toxin being a small polypeptide, devoid of large aromatic residues. Both findings were in agreement with the hypothesis that the killer toxin of *W.mrakii* acts as an ionophore on sensitive yeast strains. Purification has been hampered by the large losses of toxin activity observed during chromatographic procedures. Although it remains unexplained this may be due to partial binding of the killer factor to the chromatographic bed, or dissociation of the active components during separation.

The microtitre assay of toxin activity relied on growth, or no growth, of an indicator strain and was sensitive, therefore, to increasing salt concentrations in the elution buffers. An MTT-colourimetric assay was developed which was based on the reduction of a dye by viable cells with intact mitochondrial enzyme activity. The assay accurately distinguished between live and dead, or toxin-treated, cells of a sensitive strain of *C.glabrata*, was sensitive to increasing amounts of toxin and was able to detect toxin activity at high salt concentrations. This assay has the potential to accurately assess toxin activity and, therefore, would be important in any future purification work.

CHAPTER SIX

Mode of Action of *Williopsis mrakii* Killer Toxin Against Sensitive Yeast Strains

6.1 Introduction

Most studies which are concerned with the mode of action of yeast killer toxins have been conducted with the K1 killer protein of *S.cerevisiae*. The toxin must adsorb to a primary receptor located on the sensitive yeast cell wall (Al-Aidroos and Bussey, 1978; Bussey, 1991), but although this cell wall receptor is necessary for toxin action it does not appear to be the only component involved in the killing process. Evidence suggests the involvement of a second receptor on the plasma membrane (Bussey *et al.*, 1979). Binding at this level results in the disruption of ion gradients, interrupting the coupled transport of H^+ and amino acids (de la Pena *et al.*, 1981), and the formation of leak pathways across the membrane (Kagan, 1983).

Preliminary studies were conducted to investigate the mode of action of *W.mrakii* (K-500) killer toxin on sensitive strains of *Candida*. The effect of growth phase on sensitivity to the killer toxin and the effect of the killer factor on growth kinetics and sensitive cell viability were investigated. The possibility that K-500 killer toxin produced lethality in *Candida* strains by causing membrane damage was also investigated.

6.2 Effect of Growth Phase on Sensitivity to *W.mrakii* Killer Toxin

Initially, a profile of the growth of the indicator strain *C.glabrata* (S-388) was determined. Stationary phase cells were inoculated into fresh media and incubated at 30^{o}C, with shaking. At hourly intervals cells were removed and their numbers determined using a haemocytometer. Figure 6.1 shows a typical growth curve for the sensitive strain. Under the conditions described, cells entered an exponential phase of growth after an initial lag period of two to three hours, and then progressed into stationary phase after seven hours.

The activity of the killer factor against cells removed at the different stages of the growth cycle was assessed using a microtitre assay (section 2.11.1). 'Sensitivity profiles', showing the response to increasing amounts of killer toxin, were determined for each group of cells (Figures 6.2 A and B). Figure 6.3 further highlighted the growth inhibition produced by various concentrations of toxin assayed against cells of different

Figure 6.1 - Typical growth curve of the indicator strain *C.glabrata* (S-388). Stationary phase cells were transfered to fresh media (YNBGS) and incubated, with shaking, at 30^{0}C. Cell counts were made using a New Improved Nebauer haemocytometer.

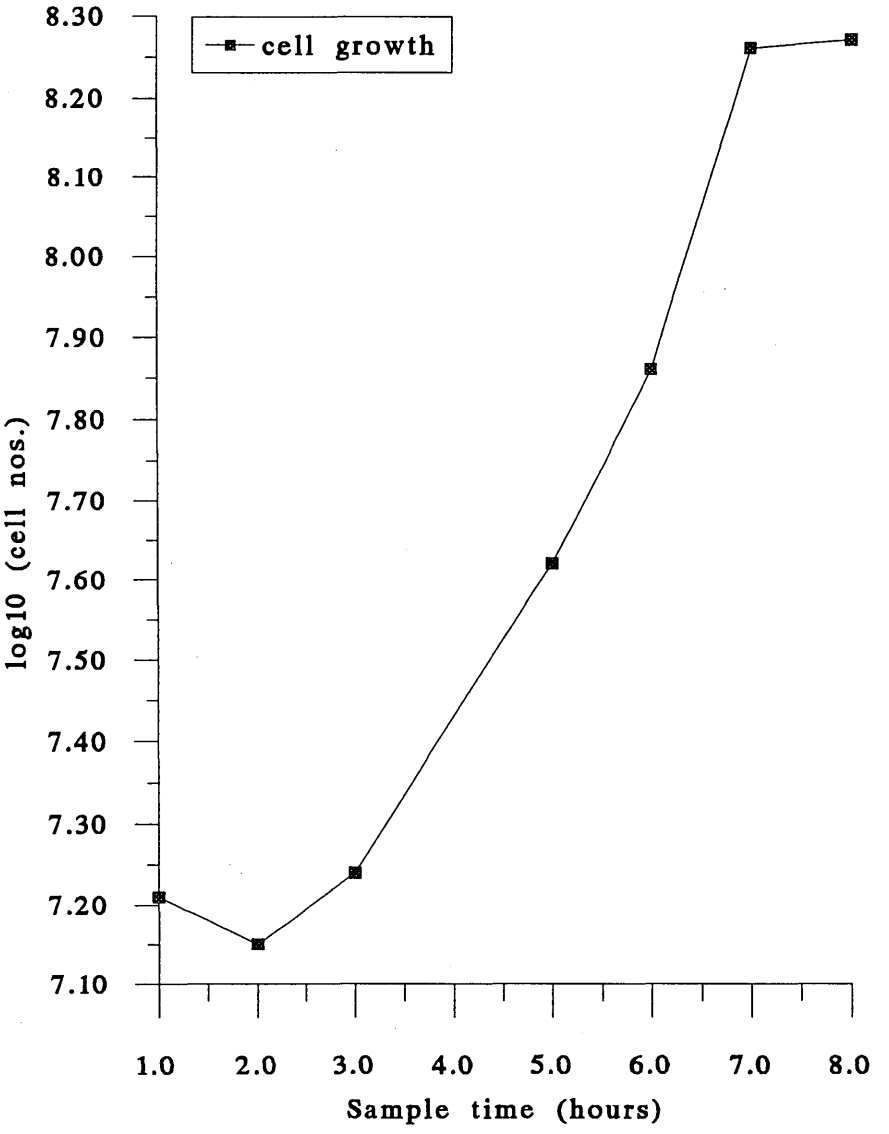


Figure 6.2 - The effect of growth phase of the indicator strain on sensitivity to *W.mrakii* (K-500) killer toxin. Cells from various stages of growth (**A**, 1-3 hours; **B**, 5-8 hours) were removed and their sensitivity to 10-100 µl of concentrated cell-free supernatant from a five day batch culture was assessed using a microtitre plate assay.

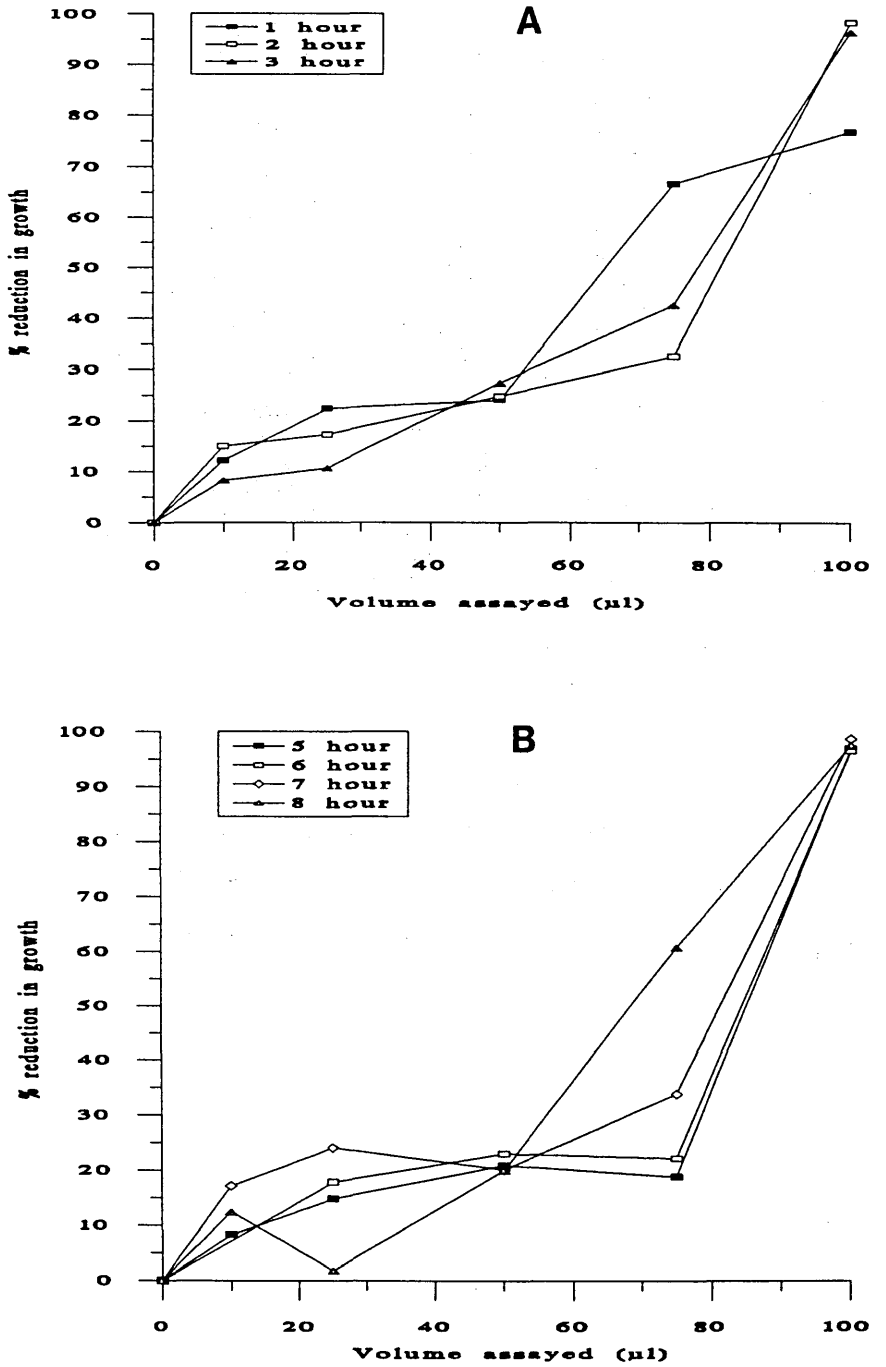
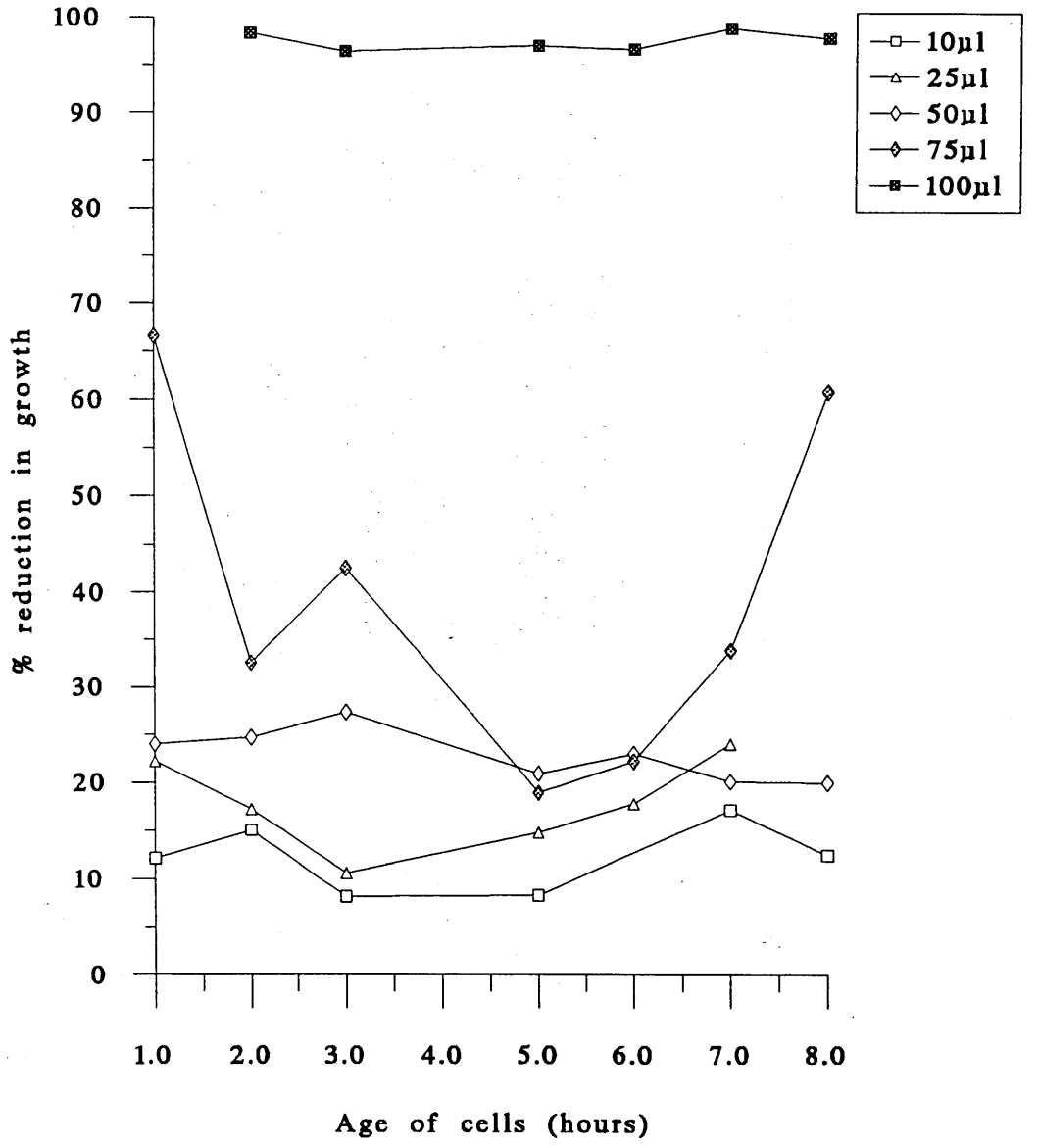


Figure 6.3 - Sensitivity of cells of *C.glabrata* (S-388) from successive stages of the growth cycle to 10-100 µl of concentrated cell-free supernatants from a five day batch culture of *W.mrakii* (K-500).



ages. At high toxin levels (100 µl) the effect on sensitive cells was the same irrespective of the phase of growth from which they were removed, in all samples complete inhibition of growth was produced. At lower toxin levels (75 µl) the effect was more pronounced, with stationary phase cells being more susceptible to toxin action than exponential phase cells. The latter, in a phase of active growth, were 30-40% less sensitive to toxin action. At very low toxin levels (25-10 µl) cells showed little response to the killer factor irrespective of their growth phase.

6.3 Effect of *W.mrakii* Killer Toxin on the Growth Kinetics of Sensitive *Candida*

The effect of *W.mrakii* (K-500) killer toxin on the growth kinetics of three strains of *C.albicans*, 2005E, 2402E and C316, was investigated. A microtitre plate assay was set up as described in section 2.11.2 and was incubated at 30^{o}C in an automated spectrophotometer. Growth of each strain, in the presence of different amounts (10-100 µl) of killer toxin, was monitored by measurement of absorbance (450 nm) at 15 minute intervals. Growth was measured over a 24 hour period and the data collated *in situ* to produce growth curves for each incubation.

W.mrakii killer toxin produced growth inhibition in three isolates of *C.albicans*. The effect of critical volumes of toxin on the growth of each isolate are displayed in Figures 6.4, 6.5 and 6.6. As the amount of toxin present in the assay system increased above a critical level, there was an increase in the lag phase before entry of the cells into an exponential phase of growth. The extent of growth, as measured by absorbance readings at 450 nm, was also reduced over a 24 hour period.

The effect of increasing amounts of toxin on the growth rate, hence, cell division, during the exponential phase of growth of each isolate was calculated (Figure 6.7). Each strain displayed a differential sensitivity to toxin action. C316 was the most sensitive and incubation with 10 µl of killer toxin directly affected the rate of cell division. Complete inhibition of growth was produced when C316 was incubated with 40 µl of toxin. Higher volumes of toxin, 40 µl and 80 µl, were required to affect the growth rates in strains 2402E and 2005E, respectively.

Figure 6.4 - The effect of critical volumes of *W.mrakii* (K-500) killer toxin on the growth kinetics of a sensitive strain of *C.albicans* (2402E). Growth was monitored over a 24 hour period using an automated spectrophotometer.

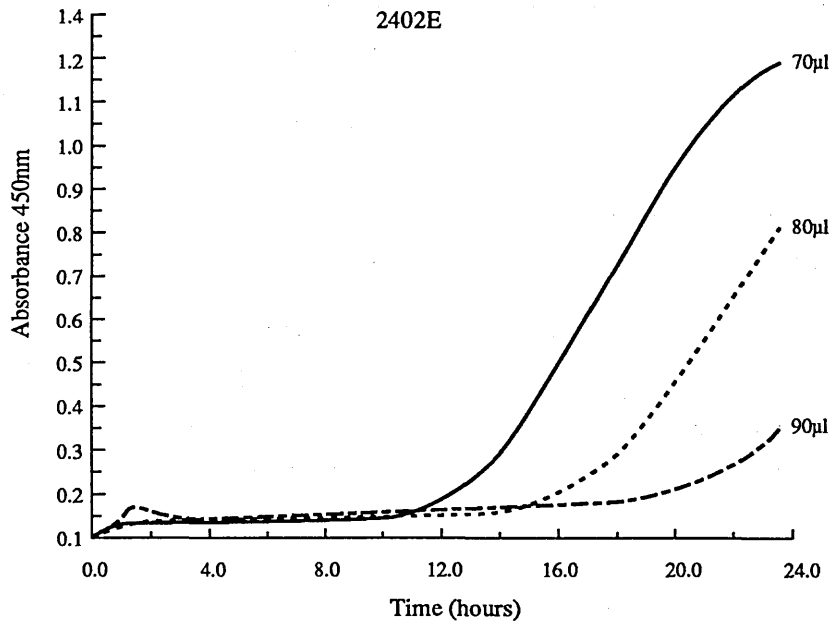


Figure 6.5 - The effect of critical volumes of *W.mrakii* (K-500) killer toxin on the growth kinetics of a sensitive strain of *C.albicans* (2005E). Growth was monitored over a 24 hour period using an automated spectrophotometer.

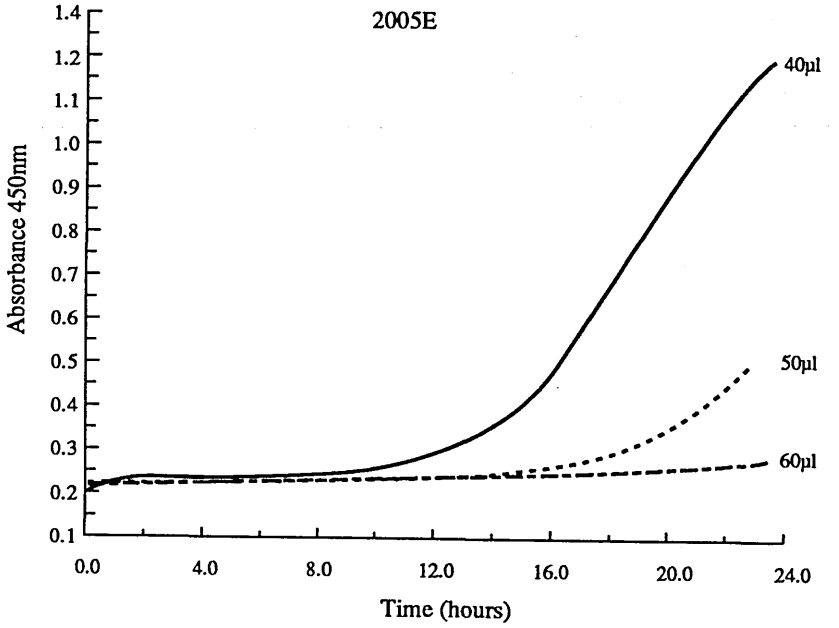


Figure 6.6 - The effect of critical volumes of *W.mrakii* (K-500) killer toxin on the growth kinetics of a sensitive strain of *C.albicans* (C316). Growth was monitored over a 24 hour period using an automated spectrophotometer.

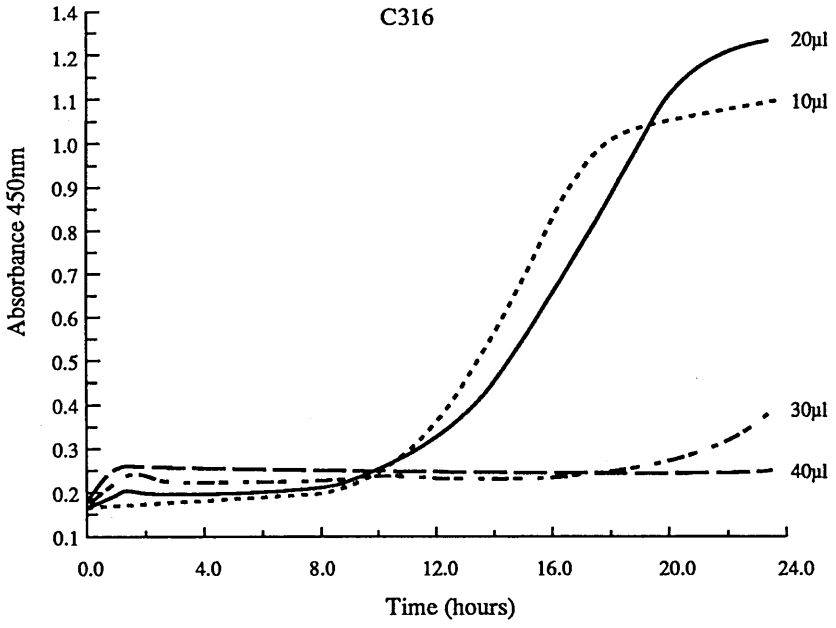
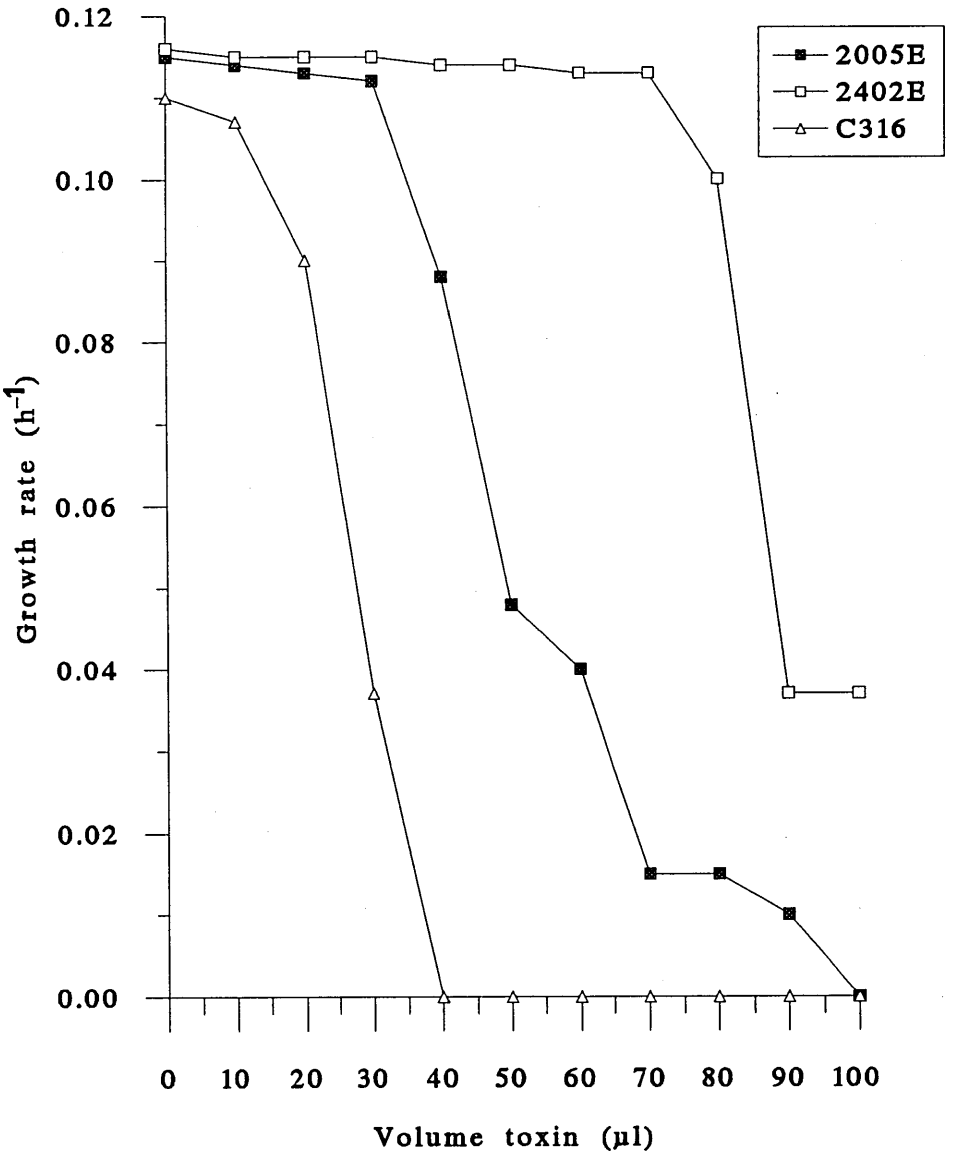


Figure 6.7 - The effect of *W.mrakii* (K-500) killer toxin on the growth rates of sensitive strains of *C.albicans*, 2402E, 2005E and C316 over a 24 hour period.



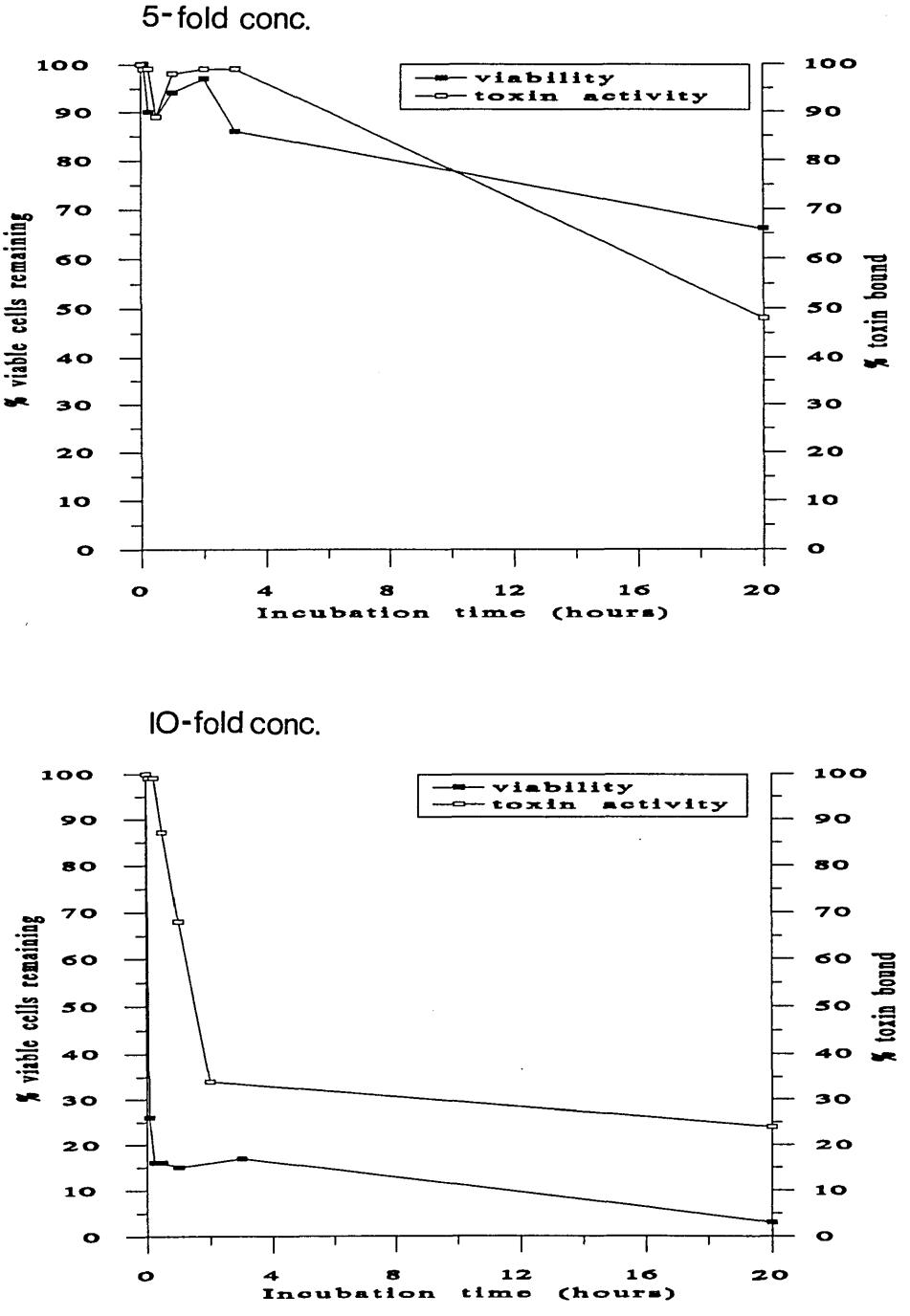
6.4 Effect of *W.mrakii* Killer Toxin on Sensitive Cell Viability

The effect of concentrated crude toxin preparations on the sensitive strain *C.glabrata* S-388, was conducted as described in section 2.11.3. Late-log phase cells of S-388 were incubated with the toxin (cell-free supernatants were concentrated five and 10-fold by freeze-drying and reconstitution in fresh media) and, at intervals, samples were removed to assess the number of viable cells which remained and also the extent of toxin binding to the sensitive cells. The latter was assessed by measurement of the residual toxin activity, after removal of the cells by centrifugation, in the supernatants in comparison to activity present in the starting material.

The first incubation of sensitive cells with the killer factor of *W.mrakii* (concentrated five-fold) showed that toxin binding closely paralleled the decrease in sensitive cell viability (Figure 6.8). A slight loss of toxin activity present in the supernatants indicated the onset of toxin binding within the first 15 minutes of incubation. After 30 minutes, 90% viable cells remained and 10% of the available toxin was bound. Between 30 minutes and 2 hours there appeared to be a slight recovery in the number of viable cells present and this coincided with an apparent loss of bound toxin from the sensitive cells. However, over the remainder of the experiment a steady decrease in sensitive cell viability occurred and 66% of the cells were viable after a 20 hour period. At this time, 50% of the original activity was bound to the cells. After 67 hours, only 6% of the remaining cells were viable and produced colonies on SDA and 34% of the initial killer activity was detectable (data not shown). To confirm that the observed decrease in toxin activity was not as a direct result of its instability under the conditions of the experiment, a separate sample of killer toxin was assayed for activity. It was found that the toxin maintained its activity over the period of the experiment.

Incubation of the sensitive cells with higher amounts of toxin (10-fold concentration) produced a more distinct effect (Figure 6.8). A small amount of toxin again bound to the sensitive cells within the first 15 minutes, however, this resulted in a 75% loss of sensitive cell viability. Within 2 hours 65% of the toxin activity was bound to the sensitive cells but with no further effect on viability. At the conclusion, only 20% of the original activity remained in solution and 3% of the cells were viable.

Figure 6.8 - The effect of *W.mrakii* (K-500) killer toxin on sensitive cell viability. Cells of *C.glabrata* (S-388), at a seeding density of $3x10^7$ cells/ml, were incubated with concentrated preparations (5- and 10-fold) of crude toxin. At intervals the numbers of viable cells were determined by colony forming activity on SDA plates and toxin binding by measurement of residual toxin activity in the supernatants.

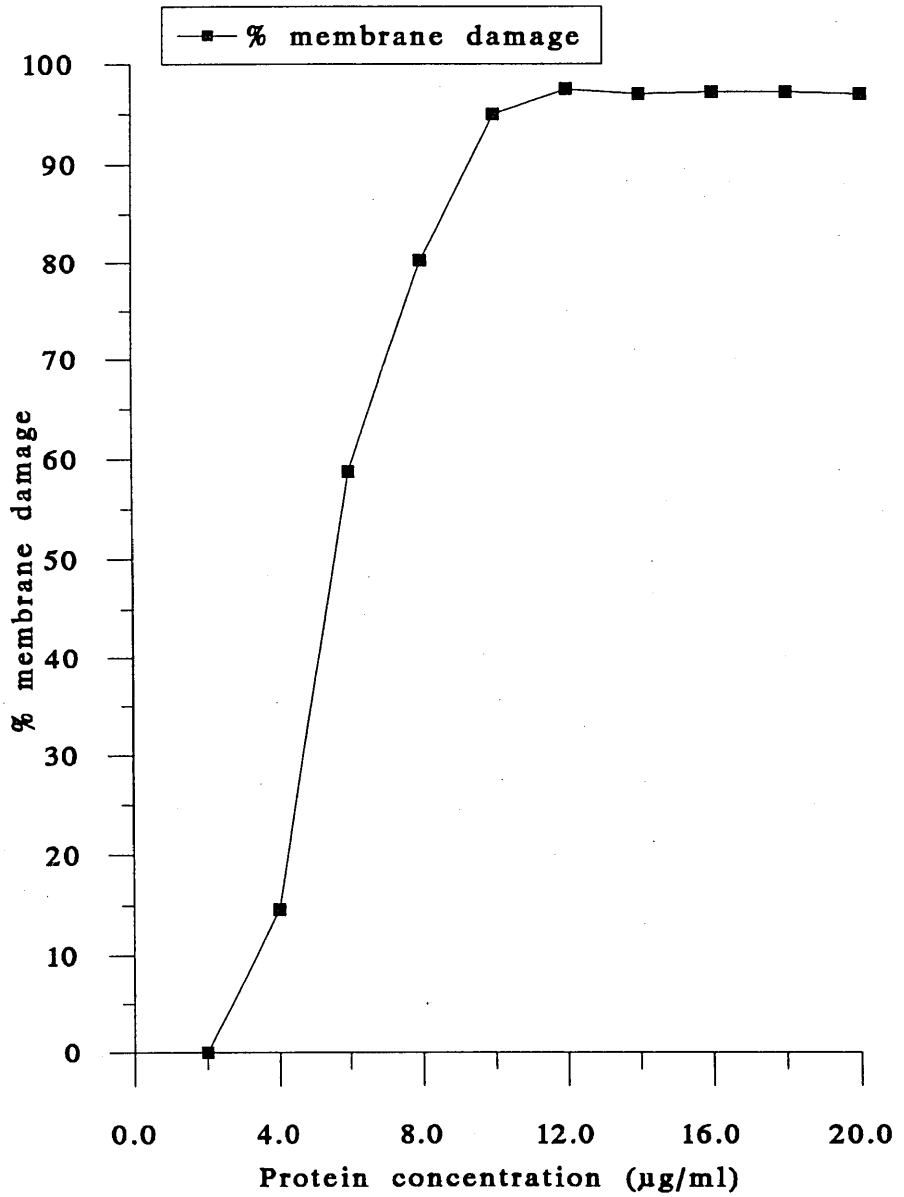


6.5 *W.mrakii* (K-500) Killer Toxin Causes Membrane Damage

A sample of crude *W.mrakii* killer toxin was tested in a membrane damage assay developed by Glaxo Group Research (see section 2.6.5). The assay involved the uptake of a radioactively-labelled non-metabolisable amino acid by actively dividing sensitive cells of *C.albicans* (2005E). Cells were incubated with toxin, or a toxin-free control (distilled water), in the wells of a microtitre plate and then harvested onto filter papers. The radioactivity of each incubation was measured and the extent of membrane damage, based on released radioactivity, was calculated with respect to the controls.

Results in Figure 6.9 showed that the extent of membrane damage caused in sensitive cells of *C.albicans*, increased as the amount of *W.mrakii* killer toxin, or protein, per well of the assay was increased. The amount of radioactive label released from the sensitive cells increased with the extent of damage caused by toxin treatment. A minimum inhibitory concentration (MIC) of 3.0 µg/ml (relevant to the amount of protein in the assay) was observed. An initial sharp increase (15-95%) in the extent of membrane damage was produced over a narrow range of toxin concentrations (5.0-10.0 µg/ml), however, no further increase in membrane damage was seen at concentrations exceeding 12.0 µg/ml.

Figure 6.9 - The effect of treatment of sensitive cells of *C.glabrata* (S-388) with *W.mrakii* (K-500) killer toxin. The extent of membrane damage produced was expressed as the amount of radioactivity released from pre-loaded cells after toxin treatment, with respect to a non-treated control.



6.6 Discussion

The mode of action and the lethal effects exerted by yeast killer toxins on sensitive cells have been investigated for only a few toxins. The K1 killer toxin of *S.cerevisiae* is characterised by leakage of K^+ (Skipper and Bussey, 1977), ATP and inhibition of macromolecular synthesis (Bussey and Sherman, 1973). The toxin of *K.lactis* causes the arrest of cell proliferation in G_1 phase of the cell cycle (White *et al.*, 1989), however, its exact mechanism of action still remains unknown. Previous studies with a strain of *W.mrakii* (LKB 169), suggested that this killer yeast produced a toxic protein which preferentially inhibited β-(1,3)-glucan synthesis (Yamamoto *et al.*, 1986b), and it was hoped that the preliminary studies undertaken would give an insight into the mode of action of *W.mrakii* (K-500) killer toxin.

The growth phase of the sensitive strain plays an important role in the killer yeast phenomenon (Polonelli *et al.*, 1991a). The killer toxin of *W.mrakii* (K-500) was more active against stationary phase cells of the indicator strain *C.glabrata*, which contradicted all previous reports (Pietras and Bruenn, 1976; Tipper and Bostian, 1984). Woods (1966) reported that sensitive cells were more susceptible during active growth but were completely resistant during their stationary phase, and similar effects have been documented for the action of the bacteriocin megacin on strains of *Bacillus megaterium* (Holland, 1962).

The susceptibility of *C.glabrata* was studied by comparing the extent of killing, or the percentage reduction in growth of the indicator strain, by killer toxin at different stages of the growth cycle. By assessing the effect of increasing amounts of toxin, 'sensitivity profiles' for each phase of cells were determined (Figure 6.2 A and B). An increase in the amount of toxin present in the assay resulted in further reductions in the growth of the sensitive yeast cells irrespective of their growth phase. All groups of cells were sensitive, to a greater or lesser degree, to the action of the killer toxin of *W.mrakii*, however, at certain toxin levels, differences in their susceptibilities became apparent. Figure 6.3 showed the effect of different amounts of toxin on cells from each growth phase. The highest amount of toxin in the assay (100 µl) produced the same effect, complete growth inhibition, in cells sampled from each stage of the growth cycle. If a

critical number of toxin molecules were required for inhibition of sensitive cell growth, it was possible that at these levels a 'saturation point' was reached where excess toxin molecules were present in relation to the number of available receptors on the sensitive yeast cell wall. At lower toxin levels (75 µl), the cells from each phase of growth showed differences in their sensitivity to the killer toxin. A 60-66% reduction in growth was produced in stationary phase cells (one and eight hour old cells), however, the same preparation produced only 20-40% reduction in exponential phase cells. This indicated that a critical level of toxin molecules had been reached, above the threshold limit of the assay (see section 5.10), which could differentiate between cells from various stages of growth. If the killer toxin of *W.mrakii*, in common with other killer yeasts, acted at the level of the cell wall, it was possible that fewer receptors were available for interaction with toxin molecules in exponential phase cells, or the receptors had less affinity for the toxin during their active phase of growth. Either of these possibilities could explain the reduction in growth inhibition observed in exponential phase cells. At lower concentrations, the levels of toxin molecules fell below the threshold limit of the assay, hence, the less pronounced effect observed.

The microtitre assay used to assess killer toxin activity displayed a threshold limit above which a very small increase in the concentration of toxin molecules present resulted in a pronounced reduction in sensitive cell growth. This suggests that more than one receptor site must be occupied on the sensitive cell for a lethal effect to be exerted, or there must be an aggregation of several toxin molecules at each receptor site.

The addition of *W.mrakii* killer toxin to cultures of sensitive strains of *C.albicans* led to an inhibition in the accumulation of cells over a 24 hour period (Figures 6.4-6.6). At critical volumes of toxin there was a reduction, or no further increase (C316, Figure 6.6) in the turbidity of the cultures. The killer toxin maintained cells in a lag phase and prevented, or inhibited, their progression into exponential growth and an increase in cell numbers. During lag phase, cells normally enlarge in size and there is extensive macromolecular synthesis. This period can be termed a 'tooling up' stage, during which growth of the cells occurs without cell division and there is an accumulation of molecules essential for cell division. Especially important are molecules of ATP which represent the supply of energy, ribosomes, required for protein synthesis, and any

enzymes which may be required for growth (Nester *et al.*, 1978). The killer factor of *W.mrakii* may cause an arrest early in the cell cycle, thereby preventing cells from enlarging and reaching the critical size necessary for continued growth and cell division. In a similar manner, the killer toxin of *K.lactis* is thought to arrest cells in the G_1 phase of growth. The addition of *K.lactis* toxin to cultures of sensitive cells of *S.cerevisiae* led to a block in the accumulation of cells and concomitant to this was a rapid and progressive loss in cell viability. Also in treated cultures, the proportion of budding cells in the population fell rapidly, and because G_1 is the only phase of the cell cycle during which cells are unbudded, this was a clear indication of arrest in G_1 after toxin addition (White *et al.*, 1989). It was not determined at this time if the cessation of cell growth by *W.mrakii* killer toxin was linked to a loss of sensitive cell viability, or if there was a direct effect on the budding process.

During the exponential phase of growth, cells divide at their maximum rate. Sub-critical concentrations of toxin led to the slowing of growth rates in all three sensitive strains (Figure 6.7), therefore, fewer cells were accumulating over the same period of time when compared to a toxin-free control. The killer toxin may have reduced the number of viable cells in the culture, therefore, fewer cells entered cell division which resulted in slower growth rates, or fewer cells had enlarged to the size at which they were ready to divide.

The dose-response of the sensitive strains to the effects of the killer toxin again supported the hypothesis that a critical number of toxin molecules were associated with, or bound to, the sensitive cell to elicit a lethal or inhibitory event. The differential sensitivity observed between the three strains of *C.albicans* may, therefore, be a result of different numbers of receptors being available on the sensitive cell wall for toxin-receptor interactions.

According to de la Pena *et al.* (1980), killer toxin from a strain of *S.cerevisiae* bound to sensitive cells immediately after addition. However, 50% mortality was produced only after 40 minutes. A lag phase before the killer toxin exerted its action had previously been reported (Kotani *et al.*, 1977; Skipper and Bussey, 1977), but de la Pena *et al.* (1981) later described an immediate effect on the co-transport of amino acids and H^+.

The early work conducted with the killer toxin of *W.mrakii* suggested that extensive growth inhibition was produced in sensitive strains of *C.albicans*, however, there was no direct evidence that the toxin produced a cidal effect. Subsequent studies showed that after initial toxin binding there was an immediate effect on sensitive cell viability (Figure 6.8), and the lag period prior to toxin action, previously reported, was not observed.

Two toxin preparations were employed to monitor the effect of *W.mrakii* killer toxin on the indicator strain *C.glabrata* (S-388), five-fold and 10-fold concentrated cell-free supernatants. Figure 6.8 shows the response of the sensitive cells to each preparation over a 20 hour period. In both cases, a minimal amount of toxin binding was apparent within 15 minutes, however, distinct differences in their effects on sensitive cell viability were observed. Binding of toxin from the more concentrated solution resulted in a 75% loss in the number of viable cells remaining within this 15 minute period. The initial response was followed by an increase (3-65%) in toxin binding over a two hour period, however, this only produced a further 10% loss in viable cells. This suggested that toxin molecules were possibly binding to dead, as well as, live cells and that the ultrastructure of the dead cells remained intact following toxin action. This allowed continued binding of the toxin to saturation.

When fewer toxin molecules were present, the effect on the sensitive cells was less pronounced. The same initial period of toxin binding led to only a 10% decrease in the number of viable cells remaining, and this was followed by an apparent recovery in both viability and the amount of toxin which was bound. The effect observed may have been the result of an intrinsic repair mechanism which existed within the sensitive yeast strain. Kotani *et al.* (1977) proposed a similar model for the action of the sake killer toxin. Cells exposed to killer toxin entered a transient state from which an irreversible reaction proceeded, this was enhanced by ATP and inhibited by Ca^{2+}. It is, therefore, possible that when low levels of toxin were bound to the sensitive yeast a repair action allowed the reversal of sensitive cells to a normal state and continued growth. However, in time sufficient toxin molecules became bound (50%) to override the repair processes and this action was coupled to a loss in viability (34%). When a comparison is made between the two toxin preparations, it can be seen that for the less concentrated solution

a longer period of time was necessary for a comparable loss in viability to occur, after which time a similar amount of toxin binding had occurred.

During a period of active research within Glaxo Group Research the killer toxin of *W.mrakii* was tested in a routine screen which assayed natural products for their ability to cause membrane damage in strains of *C.albicans*. Mid-log phase cells of the strain 2005E were incubated with the radioactively labelled 2-[^{14}C]-aminoisobutyric acid. The actively dividing cells took up the labelled amino acid which was not incorporated into protein by the cells, therefore, a 'bag of radioactive label' was produced. The loaded cells were incubated with increasing amounts of the killer factor or toxin-free controls. In the latter, the cell membrane remained intact and all radioactivity was maintained within the cells. Damaged cells, however, released the labelled amino acid because of an alteration in the integrity of the cell membrane.

Figure 6.9 shows the percentage membrane damage caused by increasing amounts of the killer toxin. Below concentrations of 3.0 μg/ml no membrane damage was caused, perhaps an indication that insufficient toxin molecules were present in the assay to affect the cell membrane. Between 5.0 and 10.0 μg/ml there was a rapid increase in the extent of damage caused as a critical number of toxin molecules attached to, and acted upon, the sensitive cells. However, at concentrations exceeding 12.0 μg/ml there was no further damage caused when a saturation point was reached. It would appear, therefore, that in common with *P.kluyveri* and *S.cerevisiae* killer toxins, that the killer factor of *W.mrakii* causes membrane damage in sensitive yeast strains.

Genetic and biochemical studies of the action of the K1 killer toxin of *S.cerevisiae* indicated a series of specific cell surface interactions. The initial step involved the binding to a β-(1,6)-D-glucan-containing cell wall receptor (Boone *et al.*, 1990). Following binding to the cell wall, lethal physiological changes occurred at the plasma membrane and intoxicated cells showed reduced proton gradients, K^+ efflux (de la Pena *et al.*, 1981) and loss of ATP and small metabolites (Skipper and Bussey, 1977). It was suggested that these effects were as a result of the toxin forming transmembrane protein channels. Kagan (1983) showed that partially purified extracts of *P.kluyveri* induced the formation of ion-permeable channels *in vitro* in a lipid bilayer membrane. The measured conductance of the channels was sufficient to account for the *in vivo* K^+, H^+ and Cl^-

efflux, and the concomitant influx of Na^+. More recently Martinac *et al.* (1990) demonstrated that the K1 killer toxin induced conductances in sensitive yeast sphaeroplasts and in artificial liposomes, and implicated these channels in the killing process. However, reports (Zhu and Bussey, 1991) that the K1 toxin killed sphaeroplasts of toxin insensitive whole cells of *C.albicans*, *C.utilis* and *K.lactis* but failed to kill those from *W.mrakii* and *P.kluyveri* suggests that perhaps other discriminatory events and components, apart from channel formation, are also necessary for the *in vivo* action of killer toxins.

6.7 Conclusions

Growth phase of the sensitive yeast strain is an important consideration in the action of yeast killer toxins. In apparent contradiction to several previous reports, the killer factor of *W.mrakii* at its critical concentration exerts a greater effect on stationary phase cells than actively dividing cells in an exponential phase of growth.

Toxin-binding experiments suggested evidence for the presence of receptors on the surface of the sensitive cell wall, however, their exact nature remains unknown. At low concentrations, the killer toxin produces a fungistatic effect on sensitive yeast but at higher concentrations a capacity to cause membrane damage accounts for the fungicidal effects of the killer factor. The cidal nature of the toxin was reflected in a rapid loss of sensitive cell viability.

The lethality produced in sensitive yeast strains by *W.mrakii* killer toxin is likely to be a receptor-mediated action in common with other killer toxins and bacteriocins. These preliminary studies give an important starting point for understanding the undoubtedly complex nature of the interaction between the killer factor and its target sensitive yeast.

CHAPTER SEVEN

Concluding Discussion

7.1 Conclusions

Early findings during this research indicated the potential of killer yeasts and their toxins to be used as 'natural antifungals' against the medically important, opportunistic pathogen *C.albicans*.

A screen of putative killer strains showed that certain yeasts caused growth inhibition, or lethality, in other 'sensitive' strains by the release of a killer factor. The killer trait was not confined solely to any one genus or species and six of the eight genera tested displayed some degree of killing against each other or known sensitive strains of *Candida* and *S.cerevisiae*. The spectrum of action observed was, generally, not directed against one genus but rather an interspecific killing action was seen. Killer strains of *S.cerevisiae*, however, predominantly showed intraspecies killing as reported by Stumm *et al.* (1977). The killer-sensitive relationships observed between the killer strains allowed a grouping of the yeasts both in terms of killer and resistance phenotypes. As a means of further classifying the yeasts it was certainly less complex than biochemical analysis of the killer factor produced by each. Certain similarities with an earlier study (Young and Yagui, 1978) were apparent. However, the conditions of the agar diffusion bioassay can easily discriminate against either the killer or sensitive strain in terms of their optimal conditions for growth and production of the killer factor. Therefore, it was not unusual for such a study to show contrasting relationships between the different yeasts screened.

The highest proportion of killer strains were found in the genera *Pichia* and *Williopsis* (formerly *Hansenula*) which is in agreement with several earlier reports (Philliskirk and Young, 1975; Kazantseva and Zimina, 1989). A strain of *W.mrakii* (K-500) consistently showed a broad spectrum of activity against a range of yeasts and the killer factor produced was lethal to several strains of *Candida*. Strains of *C.albicans* have also been shown to be sensitive to toxins produced by *Pichia* and *Kluyveromyces* (Morace *et al.*, 1984). As a consequence *W.mrakii*, and the toxin it produced, were investigated further in an attempt to provide information on a novel therapeutic approach which could help address the ever increasing problem of controlling *Candida* infections in hospitalised, or compromised individuals.

To further highlight the clinical importance of the killer factor produced, a screen against *Candida* strains isolated directly from clinical specimens was conducted. Initial

findings suggested that a differential sensitivity to the killer yeast and its toxin existed amongst yeasts isolated from disparate sites within the body. *W.mrakii* was more active against lung and tracheal isolates than vaginal isolates, implying that a niche-specific response was being observed. This study was expanded to consider a larger number of specimens and although similar trends were apparent, no clear-cut relationship between the site of isolation and susceptibility to the killer toxin was found. Similarly, no correlation was observed between biotype and specimen collection site in a study conducted by Kandel (1988), although a significantly high proportion of blood isolates were found to be resistant to toxin action. As a commensal organism *Candida* species have been isolated from almost every site in the body and if the host is predisposed to infection or there is a disruption in the balance between the host and its indigenous flora, the occurrence of certain species is heightened. It was possible, therefore, that the patterns of sensitivity observed during this work were directly related to the species of the yeasts found. Of the isolates positively identified, 84% were *C.albicans* which is by far the most proliferate yeast found in healthy or diseased individuals. These findings suggested that the killer-sensitive relationships observed were not simply dependent on the isolation site or the identity of the yeast, but more probably the result of a combination of factors. The lethality produced in a sensitive yeast strain is generally thought to involve an interaction between the toxin molecule and a receptor on the cell wall (Bussey *et al.*, 1979). The site of isolation, status of the host and species-type will all contribute to the success of this interaction. The presence of the correct receptor on the cell wall, the number present and their availability or avidity for the toxin molecules will govern the susceptibilty of any one isolate to the action of the killer factor.

Several studies were designed to further investigate the mode of action of *W.mrakii* killer toxin on sensitive *Candida* strains. It was observed that cells from various stages of the growth cycle showed a differential susceptibility to the killer factor. Contrary to several other reports (Woods, 1966; Pietras and Bruen, 1976; Tipper and Bostian, 1984) these studies suggested that stationary phase cells were more susceptible to toxin than cells in an active phase of growth. However, the differences in sensitivity were only observed at 'critical concentrations' of the killer toxin. At high concentrations, complete inhibition of growth was produced in each cell type possibly due to the saturation of all available receptor sites by excess toxin molecules. At 'critical concentrations', cells

from each phase were readily distinguishable from one another because of their different responses to the killer factor. The implication that exponential phase cells were less susceptible was perhaps due to the fact that different numbers of receptors were available for interaction at different stages of growth, or that the receptors possessed a reduced affinity for the toxin during the exponential phase. At lower concentrations, the levels of toxin molecules possibly fell below the threshold limit of the assay system which may explain the less pronounced effect observed. This in itself suggests that more than one receptor site on the sensitive cell must be occupied, or there must be an aggregation of several toxin molecules at the same site, for the threshold to be exceeded and a toxic effect produced.

A more accurate assessment of the effect of the killer factor on sensitive cell growth was made using an automated spectrophotometer which could measure cell growth constantly over a 24 hour period. A dose-response to increasing concentrations of toxin was observed, as the number of toxin molecules increased the effect on the indicator strain became more pronounced. This further supported the hypothesis that a critical number of toxin molecules were required to bind to the sensitive cell to produce a lethal effect. As toxin levels increased there was an increase in the lag phase prior to entry of the cells into their active phase of growth and a reduction in the rate of cell division leading to a decrease in final cell numbers. It was possible that the killer factor of *W.mrakii* caused an arrest early in the cell cycle in a similar manner to the *K.lactis* toxin (White *et al.*, 1989). This would prevent cells from enlarging and reaching the critical size required for continued growth and cell division. It was not determined at this stage, however, if the cessation of cell growth was linked to a loss in sensitive cell viability. If the killer toxin had reduced the number of viable cells in the culture then fewer would enter exponential growth resulting in the slower growth rates observed, or alternatively fewer cells were of sufficient size to divide. A subsequent experiment did show that the killer factor produced a cidal effect in the sensitive cells. At high toxin concentrations there was a 75% loss in the number of viable cells after a 15 minute exposure to the killer toxin, in which time only a small proportion of the toxin appeared to have been bound to the cells. In contrast, de la Pena *et al.* (1980) reported that the killer toxin from *S.cerevisiae* bound to sensitive cells immediately after addition, yet there was a lag period of 40 minutes before a 50% reduction in viability was observed. At lower toxin

concentrations a longer incubation period was required for the same effect to be seen. The results also suggested that at sub-critical concentrations of toxin the cells were able to recover from the toxic effect of the killer factor. Kotani *et al.* (1977) had previously suggested that sensitive cells entered a transition period prior to irreversible damage, during which time an intrinsic repair mechanism allowed the cells to revert to a normal state and continued growth.

The exact mechanism by which the killer toxin of *W.mrakii* exerts its lethal action on sensitive *Candida* strains remains unclear and further investigations were not possible during the time remaining. However, there is some evidence to suggest that the killer toxin produces channels in the sensitive cell which may promote the loss of ions such as K^+ and H^+ and also ATP which, ultimately, leads to cell death. The 'leak pathway' is probably the result of a secondary action of the killer factor at the level of the plasma membrane. An assay developed by Glaxo Group Research demonstrated that the toxin was in fact membrane damaging and caused the release of a radioactively-labelled amino acid from pre-loaded cells. Although this avenue of research was only given preliminary consideration it suggested that the killer toxin of *W.mrakii* acts in a similar manner to the K1 killer toxin of *S.cerevisiae* (Martinac *et al.*, 1990) and the toxin of *P.kluyveri* (Kagan, 1983).

With the knowledge that the killer factor of *W.mrakii* displayed extensive anti-*Candida* activity against clinically important isolates, it was the intention that the remainder of the research concentrated on the production and purification of the killer toxin. Any advances towards producing a novel antifungal preparation or critically assessing the mechanism of action of the toxin against *Candida* strains relied on determining the physical characteristics of the toxin and the biochemical nature of its structure. However, prior to such investigations this work concentrated on the production and processing of cultures of the killer yeast to yield workable amounts of material.

The simple nutritional requirements of the killer yeast *W.mrakii* enabled well supported growth in the minimal medium YNBGS. This chemically-defined medium was supplemented with magnesium and ammonium sulphate following the work of O'Leary (1987) in an attempt to potentiate toxin production. The lethality of the killer factor produced in the minimal medium was less than that produced in the richer YEPD

medium, but the former was used to aid processing and purification. The production of an active toxin by the killer yeast directly followed increases in cell biomass and was detectable in the extracellular medium as cells progressed through late exponential and into stationary phase. This is in agreement with earlier reports by Woods and Bevan (1968) and Palfree and Bussey (1979). Increases in the amount of protein in cell-free supernatants further confirmed production of the killer factor.

Small-scale static fermentations resulted in the production of active toxin albeit that extended periods of incubation were required to produce significant yields. Larger, agitated systems reduced the fermentation times involved and produced a more active preparation. Gentle agitation prevented pellicle formation by the yeast, maintained cells in suspension enhancing cell growth, reduced the mean generation time of cells during cell division and, it would appear, increased toxin production. Attempts to further promote toxin production under anaerobic or oxygenated conditions were unsuccessful, less toxin was produced and, in the latter, activity was lost completely. This was in all likelihood a result of the toxin being destabilised in the presence of high levels of oxygen or, alternatively, production of extracellular proteolytic enzymes was promoted under these conditions which led to inactivation of the toxin molecules. The hypothesis that the toxin structure was degraded was supported by a reduction in extracellular protein levels. Although growth of the killer yeast under anaerobic conditions was possible, reduced levels of active toxin were produced. The enhanced protein production observed in the anaerobic and oxygenated cultures suggested that not all of the protein could be attributed to active toxin. Under these conditions it was considered possible that the yeast produced significant amounts of 'stress proteins'. This area of work demonstrated that the killer yeast *W.mrakii* grew favourably in a minimal medium and was "microaerophilic" in its requirement for oxygen. Growth and toxin production were possible in the presence of only minimal levels of oxygen in a simple, chemically defined medium. It was hoped that this information would be directly applied to scale-up production of the killer factor in a 40 litre fermentation in a future collaboration with Glaxo Group Research.

A simple procedure for processing small-scale fermentations was developed which involved clarification, by centrifugation and microfiltration, and ultrafiltration steps. It was thought that the simplicity of the growth medium would benefit this procedure and

allow a rapid, partial purification of the killer factor. Preliminary findings, using PhastSystem SDS-PAGE analysis, suggested that the toxin possessed a molecular weight of approximately 16 kDa, hence, an ultrafiltration membrane with a nominal molecular weight cut-off of 10 kDa was used. Toxin activity at each stage of the procedure was assessed using an agar diffusion bioassay. The results demonstrated increasing activity *i.e.* larger zones of inhibition, in the cell-free supernatant, retentate and freeze-dried material. No activity was apparent in the permeate fraction which further indicated, at this stage, that the toxin molecule was larger than 10 kDa.

The transition from small-scale (two litres) to large-scale (40 litres) fermentations highlighted a range of technical problems involved in the production and processing of *W.mrakii* killer toxin. Gentle agitation of cultures during small-scale fermentations provided sufficient oxygen exchange for growth and the production of an active killer factor. However, the size of the vessel used, which had a 70 litre working capacity, may have created different surface:volume ratios affecting not only oxygen exchange rates but surface properties and growth patterns of the yeast. An initial fermentation supplied no oxygen directly to the system but the culture was gently agitated. As a direct consequence, cell growth over a 24 hour period was greatly reduced. Therefore, oxygen was supplied at 1.5 litres/min and a marked improvement in cell growth in the following 24 hour period was observed. The toxin activity in the cell-free supernatant after 48 hours was, however, low when compared to a static fermentation over the same period. Direct aeration of the culture in the second fermentation at a rate of 3.5 litres/min improved cell growth further, with subsequent increases in toxin production.

Toxin activity and protein levels were monitored during the downstream processing of the culture from the second fermentation, highlighting several of the difficulties encountered;

(1) significant losses of protein and toxin activity occurred during microfiltration which was purely designed as a clarification step following centrifugation. It was considered likely that the killer factor had been adsorbed to the filtration membranes as reported by Woods and Bevan (1968) and Shimizu *et al.* (1985).

(2) small losses in toxin activity and protein levels occurred during ultrafiltration, but the highest proportion of the killer factor was detected in the permeate fraction. Distribution of the killer toxin between the two fractions was not unexpected, however,

it was surprising that only a minimal amount was detected in the retentate. Initial findings concerning the size of the toxin molecules would appear at this later stage to have been misleading. Previously, permeate samples were not concentrated prior to analysis, therefore, toxin activity may have fallen below the threshold of the assay and remained undetected.

(3) dialysis of the resultant freeze-dried material, containing large amounts of unused media, removed all carbohydrate and substantial amounts of low molecular weight proteins. However, analysis of the dialysate revealed no detectable levels of toxin activity. This provided further evidence that the molecular weight was smaller than 10 kDa.

It was anticipated that this period of research would have led to the optimisation of the growth of the killer yeast *W.mrakii* and allowed the production of the killer factor in bulk for further studies. However, significant losses in toxin activity resulted during processing and only small amounts of workable material were obtained. Despite the low recovery of toxin this material remained active against other sensitive strains, including *C.glabrata* and *C.albicans*. It was considered important, therefore, within the time constraints of the project to concentrate on the characterisation and purification of the killer factor.

Although stable at low temperatures for relatively long periods of time (2-3 days at $4^{o}C$), the killer toxin of *W.mrakii* was sensitive to increasing incubation temperatures. The killer factor was stable at $37^{o}C$ for several hours but rapidly inactivated thereafter and was completely unstable to temperatures exceeding $50^{o}C$. This is unlike the killer toxin produced by another strain of *W.mrakii*, LKB 169 (Ashida *et al.*, 1983), which was stable to temperatures exceeding $100^{o}C$ for two to three minutes. The toxin maintained its activity over a range of pH 2.4-4.0 and displayed an optimal killing activity in agar diffusion bioassays at pH 3.5. No activity was detectable at values exceeding pH 5.0. In common with several other killer toxins, this information displayed obvious problems associated with the direct use of *W.mrakii* killer factor as an antifungal preparation. It would still appear feasible, however, that preparations containing the active component could be applied topically to superficial lesions rather than be used for administration, in any form, orally or intravenously. The killer was

found to be insensitive to the action of the proteolytic enzymes papain and pronase. This does not suggest that the killer factor is not proteinaceous but rather the amino acid composition is such that it may contain insufficient, or incorrect, sequences for cleavage by these site-specific enzymes, or the "cleavage" sites are buried within the tertiary folding of the toxin molecule. An alternative explanation is that other components present in the extracellular medium protect the toxin molecules from degradation or perhaps a carbohydrate moiety present in the toxin structure interferes with enzyme action. However, the inactivation of the killer factor at elevated temperatures suggests that the toxin contains a proteinaceous component necessary for its activity against sensitive yeast strains.

Preliminary studies of *W.mrakii* killer toxin involved PhastSystem SDS-PAGE and IEF analysis. Five proteins not present in the original media were detected which had distinct isoelectric points between pH 4.35 and 5.80, suggesting that the toxin was acidic. This was further confirmed by the acidification of the Coomassie blue (to yellow) present in the boiling mix used for preparation of samples for SDS-PAGE. Although the PhastSystem does not allow a critical assessment of the killer factor, it provided valuable information for future work involving FPLC purification. SDS-PAGE analysis revealed a protein of approximately 16 kDa in size. It was unusual that only a single band was detected (albeit a crude preparation) but it was possible that other proteins were present in amounts too small to be detected. No toxin activity was directly attributed to this band and several attempts to repeat this separation were unsuccessful. Significant input into the research which followed was based on these findings but with hindsight they were perhaps misleading. Subsequent studies involving gel filtration chromatography revealed that the toxin molecule may be as small as 5000-1800 Da. A modified SDS system would, therefore, be required to detect this polypeptide. The protein with an apparent molecular weight of 16 kDa was perhaps the result of an aggregation of several smaller toxin molecules. Considerable effort was expended on establishing a preliminary characterisation of *W.mrakii* killer toxin of which little has been previously reported. This study provided valuable information for continued investigations into the nature and structure of the killer factor.

A 'simple mix' of components was suggested from PhastSystem analysis of the crude toxin, therefore, use of FPLC techniques appeared to be the most profitable approach in

an attempt to purify the killer factor. With the knowledge of the isoelectric points of the toxin components a Mono Q anion-exchange column was used. As expected, the use of a buffer system with a pH of 4.0 (below the pH of the lowest isoelectric point) resulted in all of the activity being removed in the unbound fractions. At pH 6.0, a single fraction of the unbound material showed lethality against the indicator strain. The pH of the buffer system was, therefore, raised to pH 6.5 so that the active material would be separated early on the column. It appeared that the toxin was eluted on the salt gradient, however, it was difficult to accurately assign toxin activity to any one fraction because of 'salt interference' in the microtitre assay. The problems associated with analysis of the FPLC fractions at this stage required us to adopt a simpler approach to the purification of the killer factor using gel filtration techniques. Separation of proteinaceous material using this method is based on molecular weight distributions rather than on the specific charge on a molecule, therefore, this excludes the use of buffers with high pH or ionic strength which could directly affect toxin stability, or the assay system used to detect activity. A 'clean-up' step to remove low molecular weight material was proposed using a small, commercial G-25 Sephadex column (fractionation range 1-5 kDa). The active component was not eluted at the void volume as expected, but was separated on the column which immediately suggested that the molecule had a molecular weight of less than 5 kDa. The same separation profiles were consistently observed on larger, prepared columns (70 to 700 ml bed volume). With the enhanced resolution of these larger columns it was apparent that the active fractions did not directly coincide with the eluted protein peaks but lay between a protein doublet. U.V spectra of the active fractions had an A_{max} of 257 nm rather than 280 nm, similar to the phenomenon observed by Sawant *et al.*, 1989. This may be indicative of the toxin molecules lacking large hydrophobic residues, such as tryptophan and tyrosine, and further supported the hypothesis that the toxin was a polypeptide of perhaps 15-20 amino acid residues. As further confirmation of the low molecular weight of the toxin molecule the killer factor was applied to a P2 Biogel column (fractionation range 100-1800 Da). The toxin activity was eluted at, or near, the void volume of the column which suggested an approximate molecular weight of between 1800 and 5000 Da. These findings confirmed that the killer toxin of *W.mrakii* may act as an ionophore on sensitive yeast cells, causing membrane damage and the subsequent release of

intracellular components. Further purification of the killer factor was hampered by the large losses of activity observed during separation on the Sephadex columns. Only 5% of the original activity was present in the active fractions eluted from the column. It was considered possible that the toxin molecules were adsorbed to the chromatographic bed since a similar problem to that observed by Woods and Bevan (1968) with the killer toxin of *S.cerevisiae*. Alternatively, active components of the killer factor may have dissociated during the separation, or some losses were incurred during the concentration of samples for analysis. An experiment was therefore designed which demonstrated that not all of the observed losses in activity were due to the adsorption of the killer factor to the column. Forty percent of the toxin activity was unaccounted for, but it was not necessarily bound to the dextran matrix. Toxin activity may have been diluted in the washing stages and, therefore, remained undetected by the microtitre assay, or alternatively was maintained within the bed volume of the matrix. Complementation studies were conducted where all of the fractions eluted from the column were recombined and assayed for activity, however, no lethality against an indicator strain was observed. It is possible that during gel filtration chromatography the active material was separated from other components which normally stabilised the toxin structure. This could account for the large losses in activity.

Although there were problems associated with maintaining the stability of the killer factor of *W.mrakii* during purification, the low molecular weight material remained active against pathogenic yeast strains of *C.albicans*. Whilst this must have important implications for the epidemiological typing of such strains, it still remains a realistic target to exploit the interaction between the killer toxin and the sensitive yeast cell. Despite the low molecular weight of the killer factor, it is probably still too large to be used in systemic fungal therapy without eliciting an immune response because molecules as small as 1000 Da are known to produce a weak response. However, it is possible that the active component of this molecule may, when fully identified and sequenced, be useful in the design and synthesis of a synthetic derivative for use as an antifungal agent.

A consistent problem apparent during this period of research was the inability to accurately assess killer toxin activity in the fractions eluted from FPLC and gel filtration

columns. The microtitre assay was based on the growth/no growth of an indicator strain and was, consequently, susceptible to the osmotic effects of high salt concentrations and also displayed a threshold limit below which activity was not detectable. A novel assay of killer activity was formulated and developed in an attempt to overcome these difficulties and to aid any subsequent purification studies. This assay was based on the reduction of a yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) to a dark blue product MTT-Formazan by the mitochondrial dehydrogenase enzymes of living cells (Slater *et al.*, 1963). The assay was found to be able to readily distinguish between live and dead, hence, viable cells and also toxin-treated cells of a sensitive strain of *Candida*. Earlier work had suggested that *W.mrakii* killer toxin caused a rapid decrease in the viability of some strains of *Candida* and the colourimetric assay provided further confirmation of this. Toxin activity could, using this assay, be expressed as the percentage reduction in viability of an indicator strain. An experiment was also designed to investigate the deleterious effects of high salt concentrations on the assay system. A constant residual toxin activity was detected in the presence of increasing concentrations of salt, indicating the potential of this assay in analysis of fractions eluted during chromatographic techniques. The colourimetric MTT assay, previously used in the measurement of the candidacidal activity of mouse neutrophils (Ashman, 1986), was successfully adapted as a novel and rapid measure of the lethality of the killer factor of *W.mrakii* against sensitive strains of *Candida*. This method has the potential to be used as a standard assay of killer toxin activity to circumvent the use of traditional agar diffusion bioassays which are time-consuming and less quantitative.

7.2 Future work

The findings presented in this thesis provide a basis for the further development of a number of areas and the following may be important lines to follow;

(1) Purification of the killer toxin of *W.mrakii* to homogeneity using FPLC techniques (the accurate assessment of toxin activity being facilitated using the MTT-colourimetric assay developed in this current study).

(2) The use of purified material to further investigate possible host receptor responses. This would facilitate studies of the mechanism of action of the killer toxin and may enable the elucidation of potential targets for new antifungal agents.

(3) By utilising available human cell lines a number of investigations concerning the toxicity of the killer factor could be made. Furthermore, the use of animal model systems would enable preliminary data on the immunogenic response to be determined.

(4) On a more fundamental note, efforts could be directed towards a molecular and genetic study of *W.mrakii*. Contemporary techniques such as PCR-based DNA sequencing technology would allow;
(a) sequencing of the toxin molecule for comparison to sequence data bases for homology to known proteins.
(b) the development of a diagnostic probe for the killer trait.

CHAPTER EIGHT

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CHAPTER NINE

Appendices

9.1 PhastSystem Analysis

1. SEPARATION METHOD - SDS-PAGE

Sample applicator		Down at 1.1		0001 Vh	
Sample applicator		Up at 1.1		0010 Vh	
SEP 1.1	0250 V	10.0 mA	3.0 W	15°C	0065 Vh
SEP 1.2	0050 V	00.1 mA	0.5 W	15°C	0000 Vh

2. SEPARATION METHOD - IEF (3-9)

Sample applicator		Down at 4.2		0000 Vh	
Sample applicator		Up at 4.3		0000 Vh	
SEP 4.1	2000 V	02.5 mA	3.5 W	15°C	0075 Vh
SEP 4.2	0200 V	02.5 mA	3.5 W	15°C	0015 Vh
SEP 4.3	2000 V	02.5 mA	3.5 W	15°C	0410 Vh

3. STAINING METHOD - SILVER STAINING (SDS-PAGE)

Solution	Volume (per run) ml
50 % ethanol/10% acetic acid	100 ml
10% ethanol/5% acetic acid	500 ml
8.3% gluteraldehyde	100 ml
0.25% silver nitrate	100 ml
40 µl of 37% formaldehyde stock	200 ml
in 100 ml 2.5% sodium carbonate	
5% acetic acid	100 ml
10% acetic acid/5% glycerol	100 ml
Ultrapure water	500 ml

Step No.	Solution	IN-port	OUT-port	Time	Temperature
1	50% EtOH/10% HAc	2	0	2 min	50°C
2	10% EtOH/5% HAc	3	0	2 min	50°C
3	10 EtOH/5% HAc	3	0	4 min	50°C
4	8.3% glut.CHO	4	0	6 min	50°C
5	10% EtOH/5% HAc	3	0	3 min	50°C
6	10% EtOH/5% HAc	3	0	5 min	50°C
7	Ultrapure H_2O	5	0	2 min	50°C
8	Ultrapure H_2O	5	0	2 min	50°C
9	0.25% $AgNO_3$	6	0	13 min	40°C
10	Ultrapure H_2O	5	0	0.5 min	30°C
11	Ultrapure H_2O	5	0	0.5 min	30°C
12	Developer	7	0	0.5 min	30°C
13	Developer	7	0	4 min	30°C
14	5% HAc	8	0	2 min	50°C
15	10% HAc/5% gly.	9	0	3 min	50°C

Samples for SDS-PAGE analysis were prepared in Boiling Mix;

Stacking gel buffer (see below) 1.0 ml

25% sodium dodecyl sulphate polyacrylamide (SDS) 0.8 ml

β-mercaptoethanol 0.5 ml

Bromophenol blue (powder), to colour

Stacking gel buffer (pH 6.7) was prepared by dissolving TRIS base (5.9 g) and SDS (0.4 g) in 80 ml distilled water and adjusting to pH 6.7 using HCl. The solution was made up to a final volume of 100 ml with distilled water. Working aliquots were stored at 4°C.

Samples were added to an equal volume of boiling mix, heated to 100°C for 3 minutes, cooled and centrifuged at 4,500 rpm for 5 minutes prior to loading.

4. STAINING METHOD - SILVER STAIN (IEF 3-9)

Solution	Volume (per run) ml
20% TCA	100 ml
50% ethanol/10% acetic acid	100 ml
10% ethanol/10% acetic acid	500 ml
8.3% gluteraldehyde	100 ml
0.50% silver nitrate	100 ml
40 µl of 37% formaldehyde stock in 100 ml 2.5% sodium carbonate	200 ml
5% acetic acid	100 ml
Ultrapure water	500 ml

Samples were prepared by dissolving freeze-dried material in a minimum volume of distilled water. Prior to loading, solutions were centrifuged at 4,500 rpm for 5 minutes.

9.2 FPLC Analysis

EQUILIBRATION PROGRAM FOR MONO Q HR 5/5 COLUMN

Volume	Function	Value
0	conc % B	0
0	ml/min	1.5
0	cm/ml	0.5
5	conc % B	0
5	conc % B	100
15	conc % B	100
15	conc % B	0
20	conc % B	0

SEPARATION PROGRAM FOR MONO Q HR 5/5 COLUMN

Volume	Function	Value
0	conc % B	0
0	ml/min	1
0	cm/ml	0.5
0	portset	6.1
4	conc % B	0
24	conc % B	35
28	conc % B	100
32	conc % B	100
32	conc % B	0
32	portset	6.0
36	conc % B	0

All buffers were filtered through a 0.22 μm filter preferably using an all glass filtration apparatus. All samples were filtered through 0.22 μm syringe filters prior to loading. At end of run, the pumps and column were stored in 20% ethanol (filter sterilised).